Needle fungi and tree health of *Pinus radiata* in Tasmania

Istiana Prihatini

M. Sc. (Gadjah Mada University, Indonesia)

Submitted in fulfilment of the requirements for the

degree of Doctor of Philosophy

University of Tasmania

October 2014

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STATEMENT OF CO-AUTHORSHIP

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Istiana Prihatini, School of Land and Food, University of Tasmania (Candidate)

Caroline Mohammed, School of Land and Food, University of Tasmania (Supervisor)

Morag Glen, School of Land and Food, University of Tasmania (Co-supervisor)

David Ratkowsky, School of Land and Food, University of Tasmania

Tim Wardlaw, Forestry Tasmania

Author details and their roles:

Paper 1: Diversity and identification of fungi associated with needles of *Pinus radiata* in Tasmania

Located in Chapter 3

Istiana Prihatini (60%) designed and conducted the experiment, analysed the data and wrote the manuscript. Morag Glen (25%) and Caroline Mohammed (10%) contributed to experimental design, analysed the data, and edited the manuscript. As the industry partner, Tim Wardlaw (5%) advised on experimental design.

Paper 2: Multigene phylogenetic study of *Cyclaneusma* species

Located in Chapter 4

Istiana Prihatini (60%) designed and conducted the experiment, analysed the data and wrote the manuscript. Morag Glen (25%) and Caroline Mohammed (10%) contributed to experimental design, analysed the data, and edited the manuscript. As the industry partner, Tim Wardlaw (5%) advised on experimental design.

Paper 3: Survey of needle fungi in *Pinus radiata* trees with varying levels of resistance to spring needle cast (SNC)

Located in Chapter 5

Istiana Prihatini (60%) designed and conducted the experiment, analysed the data and wrote the manuscript. Morag Glen (25%) and Caroline Mohammed (10%) contributed to experimental design, analysed the data, and edited the manuscript. As the industry partner, Tim Wardlaw (5%) assisted with fieldwork and advised on experimental design.

Paper 4: Needle fungi in Tasmanian *Pinus radiata* plantations in relation to elevation and rainfall.

Located in Chapter 6

Istiana Prihatini (60%) designed and conducted the experiment, and wrote the manuscript. Morag Glen (20%) and Caroline Mohammed (10%) contributed to experimental design, and edited the manuscript. As the industry partner, Tim Wardlaw (5%) advised on experimental design. David Ratkowsky (5%) analysed the data.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published or submitted peer-reviewed manuscripts contributing to this thesis

Istiana Prihatini, School of Land and Food, University of Tasmania (Candidate)

Oct 2014

Assoc. Prof. Caroline Mohammed, School of Land and Food, University of Tasmania (Supervisor)

Oct 2014

Dr Morag Glen, School of Land and Food, University of Tasmania (Co-supervisor)

Oct 2014

Dr David Ratkowsky, School of Land and Food, University of Tasmania

Oct 2014

Dr Tim Wardlaw, Forestry Tasmania

Oct 2014

Prof. Holger Meinke, Head of School, School of Land and Food, University of Tasmania

Oct 2014

PUBLICATIONS AND COMMUNICATIONS

Journal articles:

1. Prihatini I, Glen M, Wardlaw T.J. and Mohammed C. 2014. **Multigene phylogenetic study of *Cyclaneusma* species**. Forest Pathology Volume 44, Issue 4, 299–309.
2. Prihatini I, Glen M, Wardlaw T.J. and Mohammed C. 2014. **Diversity and identification of fungi associated with needles of *Pinus radiata* in Tasmania**. Southern Forests (accepted subject to revision, currently under revision).
3. Prihatini I, Glen M. Wardlaw T.J. and Mohammed C. 2014. **Survey of needle fungi in *Pinus radiata* trees with varying levels of resistance to spring needle cast (SNC).** Forest Pathology (accepted subject to revision, currently under revision).
4. Prihatini I, Glen M, Wardlaw T.J. Ratkowsky D.A. and Mohammed C. 2014. **Needle fungi in Tasmanian *Pinus radiata* plantations in relation to elevation and rainfall**. New Zealand Journal of Forest Science (submitted, currently under review).

Conference proceedings:

1. I. Prihatini, A.H. Smith, J.M. Gonnet, T.J. Wardlaw, C. Beadle, M. Glen and C.L. Mohammed, 2007. **Linking environmental stress in pine plantation to fungal attack**, 16th Biennial Australasian Plant Pathology Society Conference, 24 - 27 September 2007, Adelaide, Australia, poster presentation.
2. I. Prihatini, M. Glen, A.H. Smith, T.J. Wardlaw and C.L. Mohammed, 2009. **Survey of the needle fungi associated with Spring Needle Cast in *Pinus radiata***, 17th Biennial Australia Plant Pathology Society Conference, 29 September - 1 October 2009, Newcastle, Australia, poster presentation.
3. M. Glen, I. Prihatini, A. Smith, C. Mohammed and T. Wardlaw, 2011. **Spring needle cast in Tasmania—fungal communities and environmental factors**, Inaugural joint 4th Asian Conference on Plant Pathology and the 18th Biennial Australasian Plant Pathology Society Conference, 26–29 April 2011, Darwin, Australia, oral presentation.

ABSTRACT

Pinus radiata is one of the most important species of forestry plantation worldwide and many pests and diseases have followed the expansion of this species around the globe. Tasmanian *P. radiata* plantations suffer from a needle cast disease referred to as spring needle cast (SNC) which has similar symptoms to *Cyclaneusma* needle cast, a serious disease in New Zealand. Stands with moderate or severe SNC can be expected to suffer potential losses in clear-fall volume. The aetiology of SNC is poorly understood and has long been a point of debate between Tasmania and New Zealand foresters with the New Zealanders attributing SNC to *Cyclaneusma minus*. Tasmanian research in the 90s proposed that SNC is caused by one or a suite of fungi that are triggered by an unknown environmental stress. Three species of fungi have been putatively associated with SNC, i.e. *Lophodermium pinastri*, *Strasseria geniculata* and *C. minus.*

Understanding endophyte diversity is crucial for determining their role in forest health. Endophytes are increasingly recognized for having a role in plant-pathogen interactions leading to disease. In particular, they may play an important role in determining the presence of a disease such as SNC, which does not appear to have a single pathogenic causal agent and which may be associated with environmental stress. The main objective of this study was therefore to identify needle fungal communities which are associated with both healthy and SNC-affected trees in *P. radiata* plantations in Tasmania. Needle fungi have been extensively studied in conifers but rarely in *P. radiata*.

Previous studies in *P. radiata* have mainly been based on fungal isolation. In this study, fungal communities were characterized by various detection methods including the isolation of pure cultures, morphotype grouping of cultures supported by the DNA sequencing, direct DNA analysis from needle tissue and DNA sequencing of cloned PCR products. A total of

544 isolates were obtained from 180 needle samples. The cultures were grouped according to macro-morphological characters, resulting in 26 morphological groups that each consisted of at least three cultures and 14 singletons. After phylogenetic analysis, the appropriate identity of each Operational Taxonomic Unit (OTU) was confirmed which resulted in 48 OTUs, including five basidiomycetous OTUs. The starting point of 98% sequence similarity for grouping of Molecular Operational Taxonomic Units (MOTUs) produced 130 MOTUs from 1303 sequences derived from DNA of fungal isolates and DNA extracted from *P. radiata* needles. After further analyses, a total of 154 MOTUs were recognised from isolates and DNA fragments amplified from pine needles. Only 10 of the 154 MOTUs were detected by direct DNA amplification and fungal isolation, 35 by isolation only, and the remaining 129 were detected by DNA only. The most frequently detected group of fungi detected from *P. radiata* needles in Tasmania was Dothideomycetes. Leotiomycetes was the second most frequently detected group, by culturing as well as by DNA amplification. Sordariomycetes was the third most frequently detected group, but the most highly represented by isolation. Most of the commonly isolated fungal species and many of the commonly detected MOTUs were consistent with previously known fungal associates of *P. radiata* or other pine or conifer species, e.g. species of *Lophodermium, Cyclaneusma* and *Coniochaeta*. PCR and DNA sequencing, however, revealed a number of common endophytes not previously recorded from *P. radiata* e.g. species of the Teratosphaeriaceae. All fungal detections carried out during the thesis studies provide a reference collection and DNA database of *P. radiata* needle fungi comprised of both endophytes and pathogens. Isolates and the DNA database can be further mined to investigate the potential role of endophytes in needle cast diseases in Tasmania and elsewhere in the world, especially those that might be manipulated for biocontrol.

Two different morphotypes of *Cyclaneusma* cultures from Tasmania and mainland Australia were isolated for inclusion in the phylogenetic analysis of *Cyclaneusma*. The two clades of *C. minus* were consistent with the two different morphotypes of *C. minus* reported in a previous study (Bulman and Gadgil, 2001). This morphological variation prompted a phylogenetic analysis to ascertain whether or not these two distinct morphologies represented two separate species. Analysis of five gene regions including Beta-tubulin, elongation factor and ribosomal DNA (mitochondrial large sub-unit, nuclear large sub-unit and internal transcribed spacer) sequences all supported the distinction of two cultural morphotypes (i.e. *Cyclaneusma minus* 'simile' and 'verum') as separate species. One morphotype ('verum') was more closely related to *C. niveum* than to the other morphotype ('simile'). A formal description of the new species represented by the 'simile' clade is now required. Confirmation of the occurrence of two *Cyclaneusma* species on *P. radiata* in Australia and New Zealand is a significant scientific step as the molecular characterisation of the two *Cyclaneusma* species will facilitate examination of the role each plays in needle cast diseases, especially as we are faced with an increasingly changing climate.

Previous studies have showed genetic variation in susceptibility to SNC. Pine needles representing healthy and SNC-affected trees were collected from a genetic field trial that had been established at Oonah, Australia in 1992. The fungal communities associated with healthy and SNC needles were examined by amplification, cloning and sequence analysis of the fungal DNA present in the pine needle samples. Significant differences in fungal communities were observed between healthy and SNC-affected trees. For example, some fungal species were detected more frequently in healthy than in diseased trees. Fungal communities were not markedly affected by the genetic background of the host. Of the three fungi that were previously considered likely to play a role in SNC, only *L. pinastri* was implicated.

Pinus radiata trees were scored for SNC damage in a resistance trial at Oonah in Tasmania. The Oonah study showed that trees with contrasting levels of disease severity of SNC have significantly different fungal communities. Healthy and moderately healthy trees could be separated from diseased and severely diseased trees based on their fungal communities. Seventeen species of hitherto unknown members of the Teratosphaeriaceae were identified; Teratosphaeriaceae sp. 3, Teratosphaeriaceae sp. 13, Teratosphaeriaceae sp. 24 and *L. pinastri* were the dominant OTUs more strongly associated with unhealthy trees. In healthy trees, Ascomycete sp. 2, *Phaeotheca fissurella*, Pleosporales sp. 1, *Dothistroma septosporum,* and Teratosphaeriaceae sp. 4 were the dominant OTUs within the fungal community. Out of the three species suggested as possible causal agents of SNC, only *L. pinastri* showed a greater prevalence in higher disease classes. *Strasseria geniculata* and *C. minus* 'verum' were present but in a small number of samples. Though *C. minus* was detected at moderate frequency in the current study, its frequency of detection was correlated only to host family and not to disease class or needle age. The involvement of multiple fungal species in maintaining healthy trees or in causing disease cannot be either ruled in or out by the results at Oonah e.g. an interaction between the pathogen *L. pinastri* and the presence or absence of other endophytic fungi. However, the study raises important questions about the relationship between *L. pinastri*, endophytic members of the Teratosphaeriaceae and SNC in *P. radiata*.

Needle fungal communities present in young 5-year-old *P. radiata* trees growing at different sites were strongly defined by presence or absence of pine associates that are 'true' endophytes such as species of the Teratosphaeriaceae. However, needle fungi that have been previously associated with pathogenic behaviour (species of *Dothistroma*, *Strasseria*, *Cyclaneusma* and *Lophodermium*) were identified from the young *P. radiata* but neither *D. septosporum* nor *S. geniculata* were found at the two sites where SNC was later reported

to be at a serious level. *Cyclaneusma minus* was present at one sites only. In contrast, *L. pinastri* was an almost ubiquitous needle fungus. Rainfall was the key factor correlating with fungal assemblages across the 12 sites. In forest health surveys subsequent to this study, only the two wettest sites (Inglis River and Oonah) were reported to have a serious level of SNC. This study, conducted in plantations of an age that predates the expression of SNC, showed that *L. pinastri* was ubiquitous in needles at all sites at which the climate and/or environmental stress is appropriate for disease to develop. One species, labelled Teratosphaeriaceae sp. 03, was present at only three of the 12 sites, but serious SNC subsequently developed at two of these sites. Another plausible scenario is that the causal agent of SNC is *L. pinastri* acting in conjunction with Teratosphaeriaceae sp. 03 when conditions favouring disease are present. These results with young pine support the interpretation of data from the Oonah trial i.e. *L. pinastri* is more closely associated with SNC than *C. minus* and *S. geniculata*.

In summary, the research in this thesis:

- Established a reference collection and DNA database of *P. radiata* needle fungi comprising of both endophytes and pathogens. Isolates and the DNA database can be further mined to investigate the potential role of endophytes in needle cast diseases in Tasmania and elsewhere in the world, especially those that might be manipulated for biocontrol.
- Elucidated the taxonomy of the *Cyclaneusma* isolates present in Tasmania and New Zealand. The differentiation of isolates into two species will support investigations of the pathogenic behaviour of each *Cyclaneusma* species and their interactions with endophytes. As climate changes, the role or predominance of each species in causing disease may also change.

- Determined that the causal agent of SNC in Tasmania is a ubiquitous needle fungus *L. pinastri*, which may act in conjunction with Teratosphaeriaceae sp. 03 when stressful conditions are present.

ACKNOWLEDGEMENTS

Alhamdulillah, finally I finish my doctoral thesis. I would like to thank all people who have helped and inspired me during my doctoral study at the University of Tasmania in Hobart and completing it in Indonesia. I would never have been able to do this without the guidance of my committee members, help from friends, and support from my family.

First and foremost I would like to express sincerest gratitude to my supervisors, Assoc. Prof. Caroline Mohammed and Dr. Morag Glen who have supported me with their excellent guidance, caring, patience, knowledge and providing me with an excellent atmosphere for doing research. Special thanks go to Dr. Chris Beadle, who was willing to participate in correcting my thesis, Prof. David Ratkowsky, who helped with statistical analysis and the use of Primer and Permanova software.

I would also like to thank Dr. Tim Wardlaw, Dr. Anna Smith and David Page who gave invaluable help and guidance in the field work, Adam Smolenksi who assisted me in the Molecular Genetics laboratory at the University of Tasmania. In my daily work I have been blessed with friendly and cheerful people at the Cooperative Research Centre for Forestry and CSIRO Sustainable Ecosystems, my next-door colleague, Dr. Alieta Eyles who was always willing to help and give her best suggestions, Di Tambling and Jan Ellis were always there with a friendly smile and brightened my days especially at teatime and lunchtime.

Thanks also go to a group of fellow students, Genevieve Gates, Mila Sulistyawati, Luci Agustini and Bryony Horton; it would have been a lonely lab to work in without them. My gratitude goes to all of my colleagues in Molecular Genetic lab in Yogyakarta, Indonesia who have always supported me and given me ‘room’ to finish my thesis off, special thanks to Dr. Anto Rimbawanto who constantly encourages me do to the best. Thanks to all of Indonesian people in Hobart who I cannot name one by one, to Ifayanti Ridwan Saleh and her family who helped me and my family, during the hard times in the first year of living in Australia.

I would like to thank my sponsors; my research was supported by a scholarship from the Australian Centre of International Agricultural Research (ACIAR) and operating from the Australian Research Council. Sharon Harvey of ACIAR was there to help me and make sure that my studies went well.

I would like to thank my family; my mum who is no longer with us, she worked really hard to make my dreams come true and never complained about the hardship in her life; my late

father who freely gave his love and was my good friend; my sisters and my brother who support me and encourage me with their best wishes; my daughters who cheer me up and finally my husband who has always stood by me through the good times and bad.

TABLE OF CONTENT

List of Abbreviations

βT-1	βeta-tubulin 1
CNC	Cyclaneusma Needle Cast
DNB	Dothistroma Needle Blight
ITS	Internal Transcribed Spacer
ML	Maximum Likelihood
MOTU	Molecular Operational Taxonomic Units
MP	Maximum Parsimony
mt-LSU	Mitochondrial Large Sub Unit
n-LSU	Nuclear Large Sub Unit
OTU	Operational Taxonomic Units
PNB	Physiological Needle Blight
rDNA	ribosomal DNA
RNC	Red Needle Cast
SNC	Spring Needle Cast
tef-1	translation elongation factor

I. INTRODUCTION

I.1. IMPORTANCE OF *PINUS RADIATA*

Pinus radiata (commonly referred to as radiata pine) is an exotic conifer planted in Australia (Gavran 2012), and contributes significantly to Australia's economy (ABARES 2012). Radiata pine may be used for a wide range of purposes, including light construction, furniture, panelling, internal flooring, mouldings, joinery, veneers and pulpwood. It is easy to grow, with rapid growth and is generally managed over a 25-35 year rotation (Turner and Lambert 2013). It is the only pine species grown in Tasmanian plantations, covering 75 000 ha plantation estate in 2010 (Gavran 2012).

Pinus radiata is also the most widely planted commercial species in other states of Australia (Western Australia, South Australia, New South Wales, Victoria). Although other *Pinus* species, such as *P. canariensis* and *P. palustris*, produce a better quality timber, slow growth makes them unprofitable (Spencer 1995). *Pinus radiata* is planted as the main species in New Zealand where it comprises 90% of total plantations (NZFOA 2012). Chile has 1.5 million ha of *P. radiata* plantations (Álvarez et al. 2012) which cover about 63% of total forest plantation estate (Mead 2013). *Pinus radiata* is a significant species in forest plantations in Spain and South Africa (Mead 2013).

I.2. PROBLEMS IN *P. RADIATA* PLANTATIONS

Forest health is influenced by abiotic factors such as fire, temperature, water and nutrient availability and/or biotic factors such as insect, fungal and mammal pests. The threat of pests to forest plantations applies at any stage in the rotation, from planting time to established plantations near harvest. Pests, diseases and their propagules are vectored all over the world

through pathways such as germplasm movement or the importation of wood and forest products (Webber 2010). As forestry plantations expand in many countries and with increased global trade and rapid movement of goods and people, the problems of pests and diseases in these resources have grown exponentially (Webber 2010). For *P. radiata*, two of the most recently emerging new disease threats are caused by species of foliar *Phytophthora*. In Chile, a new species of pathogen associated with dying *P. radiata* needles was confirmed as *P. pinifolia* (Duran et al. 2008), while in New Zealand, Red Needle Cast (RNC) is an emerging disease threat of *P. radiata* in New Zealand that is caused by *Phytophthora* taxon '*pluvialis*' (SCION 2013) .

Pest damage in *P. radiata* can be significant. For example, the losses caused by the aphid *Essigella californica* were estimated at approximately AUD $21M per year in Australia (May 2004). Productivity of *P. radiata* can also be dramatically reduced if needles are infected by fungal pathogens; in New Zealand, *Dothistroma septosporum* can halve productivity (O'Hehir and Nambiar 2010) while *Cyclaneusma minus* cause economic losses of around NZD $38M per year (Watt et al. 2011a).

With climate change, the impact of pests and diseases on trees is likely to change as the damage they cause to their hosts has been shown to be correlated to climatic factors such as rainfall, humidity and temperature (Tubby and Webber 2010). There have been several studies that suggest this will be the case with *P. radiata*. For instance, a modelling study demonstrated that climate change may potentially increase the risk of pitch canker incidence caused by *Fusarium circinatum* (Ganley et al. 2009, Ganley et al. 2011). Sphaeropsis shoot blight is predicted to increase as the frequency and severity of drought events increase over the next 30 years and by contrast will reduced in the next 40 years (Fabre et al. 2011). The prediction of dothistroma needle blight (DNB) under future climate varied widely throughout

New Zealand from moderate to high risk (Watt et al. 2011b). This pathogen has been already reported to be responding to climate change in British Columbia (Sturrock 2012).

I.3. NEEDLE CAST DISEASES OF *P. RADIATA* PLANTATIONS

Many different needle diseases are problematic worldwide in conifers (Bednářová et al. 2013). Examples include red needle blight caused by different species of *Dothistroma* (Barnes et al. 2004); and Swiss needle blight in Douglas-fir (*Pseudotsuga menziesii*) plantations caused by the fungus *Phaeocryptopus gaeumannii* (Maguire et al. 2011, Morales et al. 2012). *Dothistroma septosporum* causes serious damage in *P. radiata* in New Zealand (Watt et al. 2009) and in Australia's mainland plantation estate (Ivkovic et al. 2010). Damage in Tasmanian plantations is less serious (Podger 1984).

Cyclaneusma species have been reported in at least 14 species of *Pinus* (Crowley, 2006), causing significant economic losses in *P. sylvestris* in Pennsylvania, USA (Kistler and Merril 1978, Merrill and Wenner 1996) and in *P. radiata* in Spain (Magnani 1972), Chile (Luisi et al. 1987) and New Zealand (Bulman 1988, Dick 1989). In Australia, this fungal pathogen has been reported from NSW, ACT (Stahl 1965, Choi and Simpson 1991, Crowley 2006), Victoria, Tasmania (Podger and Wardlaw 1990a) and South Australia (Pawsey 1967) but the economic losses from cyclaneusma needle cast (CNC) have not been determined for Australia and Tasmania.

In Tasmania, spring needle cast (SNC) of *P. radiata* is defined by the early casting of pine needles which starts following the development of yellow and brown mottling, the symptoms starting in early spring. The disease appears in young pine plantations at canopy closure (age 6-7 years) and is manifested by the rapid browning of one-year-old needles followed by the heavy casting of needles. It is especially common in wet areas at high altitude

(Podger and Wardlaw 1990b). Spring needel cast leads to growth reductions in direct proportion to the amount of defoliation. Stands with moderate or severe SNC can be expected to suffer potential losses in clear-fall volume of 30-50% (Podger and Wardlaw 1990a). It is thought to be caused by a suite of endophytic fungi that are triggered into secondary pathogenic activity by an environmental stress (Elliot and Wardlaw 2003, Wardlaw 2008). The three fungal species that have been putatively associated with SNC in Tasmania are *Cyclaneusma*, *Strasseria* and *Lophodermium* (Podger and Wardlaw 1990a, Wardlaw 1994). The fruiting bodies of these fungi have been occasionally observed (Wardlaw 2008). In New Zealand, Bulman and Gadgil (2001) separated *C. minus* into two different morphotypes i.e. *C. minus* 'verum' and *C. minus* 'simile' according to mycelium colour, colony texture, pigmentation, sporulation and growth *in vitro*.

I.4. PINE DISEASE MANAGEMENT AND RESISTANCE

Several management practices to control needle cast diseases have been applied in pines. Copper oxychloride and cuprous oxide have been the most widely used fungicides to prevent the spread of dothistroma disease in New Zealand and Australia (Bradshaw 2004, Bulman et al. 2004). Fungicides have also been applied to control *C. minus* in *Pinus sylvestris*, but repeated applications are required and the interactions with genetic factors need to be assessed (Merrill and Wenner 1996). In New Zealand, aerial applications of fungicides have been unsuccessful in controlling CNC (Vanner 1986). Systemic fungicide injection into trees have showed some success (Hood and Vanner 1984) but this method is not feasible for large-scale plantations (Bulman 1993). In Tasmania, the application of the fungicide, chlorothalinol by spraying at six-weekly intervals resulted in increased needle retention when compared to other fungicides of SNC-affected needles (Podger and Wardlaw 1990b). However, this regime of spraying is neither cost effective nor environmentally feasible for *P. radiata* plantations.

Cultural practices (e.g. wide spacing and weed control) that minimize conditions which predispose host to disease such as free moisture may help to limit the spore germination of pine needle pathogens. However, the selection of resistant trees through breeding programmes is generally considered the most effective way to manage needle casts (Bulman et al. 2008). Variations in the susceptibility to *C. minus* have been found in various host tree species, e.g. *P. sylvestris* in the USA (Merrill and Wenner 1996) and *P. radiata* in New Zealand (Beets et al. 1997; ref?).. Populations of *P. radiata* in New Zealand appear more resistant to CNC than Californian populations (Burdon et al. 1992). In the case of SNC, there is also clear evidence that resistance to this disease is inherited, thus breeding selection programs may offer the best solution to reduce its impact in the long term (Podger and Wardlaw 1990b).

Host tree resistance is a manifestation of defence mechanism which prevents or slows down the invasion and establishment of pathogens (Bonello et al. 2006). Constitutive defense mechanisms are already present in the plant and include physical and chemical barriers (Franceschi et al. 2005, Verne et al. 2011). Defense mechanisms may also be induced in response to pathogen attack (Eyles et al. 2010) and non-pathogenic organisms such as endophytic fungi (Ganley et al. 2008).

There is increasing interest in how endophytes or fungal communities influence disease resistance in plants (Arnold et al. 2003, Rodriguez et al. 2009, Aly et al. 2011). Previous studies have found the interaction between endophyte fungi with herbivory resistance in *Populus tremula* (Albrectsen et al. 2010). An association between the presence of endophytic fungi and the status of health in confers has been reported by several authors (Giordano et al. 2009, Botella et al. 2010, Gordon and Gehring 2011). For example, endophytes have been reported to increase the survival of *Pinus monticola* from a disease caused by *Cronartium ribicola* (Ganley et al. 2008).

I.5. IDENTIFICATION OF PINE NEEDLE FUNGI USING DNA MARKERS

Pine needle fungi can be identified by the morphological characteristics of their fruiting bodies as well as their cultures or by molecular-based characters. However, many of the endophytic fungi associated with pine needles do not produce spores in culture and identification using cultural characteristics is impossible (Ganley 2004a). The recent advances in DNA sequencing technology have led to widespread adoption of DNA sequence analysis as the preferred method for fast and routine identification of fungal isolates including endophyte fungi (Ko et al. 2011). Identification of fungi has also been facilitated by the sequencing of fungal ribosomal RNA genes (Bruns and Gardes 1993). Molecular analyses may also provide additional valuable information about fungal communities or populations that can be applied to developing strategies for disease management (Udayanga et al. 2011).

I.6. PURPOSE OF MY RESEARCH

The overall objective of my study is to improve the management of needle cast diseases in *P. radiata* plantations by contributing to the knowledge of needle fungal ecology. I focus on *P. radiata* plantations in Tasmania and the needle fungi associated with the expression of SNC. My study aims to determine the identities of the individual pathogens and endophytes present in needles as well as examine the contribution of pathogens and endophytes at the fungal community level. My PhD studies will be based on four major studies: (i) a survey of the fungi associated with *P. radiata* needles in Tasmanian plantations; (ii) a multigene phylogeny of the *Cyclaneusma* species present in Tasmanial; (iii) the identification of needle fungi present in trees with and without symptoms of SNC; (iv) a study exploring the effect of envioronmental conditions on the compoisition of needle fungi associated in young radiata pine plantations.

I.7. THESIS STRUCTURE

This thesis is composed of four experimental papers. The first paper describes a survey of the fungi associated with *P. radiata* needles in Tasmanian plantations and establishes a reference collection of cultures from Tasmania, mainland Australia and New Zealand. In this paper, fungal identification was achived by employing a direct PCR approach. This paper has been submitted to Southern Forests journal and currently under revision.

Prihatini I., Glen M., Wardlaw, T.J. & Mohammed C. 2014. Diversity and identification of fungi associated with needles of Pinus radiata in Tasmania. Southern Forests Journal (accepted subject to revision, revision underway prior to resubmission).

The second paper is a multigene phylogeny study of *Cyclaneusma* to characterise the fungal species present in Tasmania. The results confirmed the identities of ttwo morphotypes of *C. minus* in Australia, which is consistent with the two morphotypes reported in New Zealand. The possibility of more than one species within *C. minus* was examined by phylogenetic analysis using sequences from five gene regions, i.e. ribosomal DNA (rDNA) Internal Transcribed Spacer (ITS), mitochondrial rDNA LSU (mt-LSU), nuclear DNA LSU (n-LSU), translation elongation factor 1 (*tef-1*) and beta tubulin 1. This paper has been published by Forest Pathology.

Prihatini I., Glen M., Wardlaw T., & Mohammed C. 2014. Multigene phylogenetic study of Cyclaneusma species. Forest Pathology Volume 44, Issue 4, pages 299–309.

The severity of SNC can vary significantly among trees growing within the same plantation. The third paper investigates the needle fungal communities (pathogens and endophytes) of *P. radiata* trees with varying levels of resistance to SNC. Correlations between particular fungal species or communities, high or low disease levels and/or host pedigree were established. The

causal agents of SNC and the possible role of endophytes in resistance are discussed. This paper has been submitted for publication by Forest Pathology and currently under revision.

Prihatini I., Glen M., Wardlaw T., & Mohammed C. 2014. Survey of needle fungi in Pinus radiata trees with varying levels of resistance to spring needle cast (SNC). Forest Pathology (accepted subject to revision, revision underway prior to resubmission).

The fourth paper characterises the needle fungi associated with young radiata pine plantations in which symptoms of SNC have yet to be expressed. The specific relationships between site environmental conditions as defined by rainfall and altitude, and the composition of fungal communities were determined. The influence of environmental conditions on fungal community composition and SNC development of older trees after canopy closure is discussed. This paper has been submitted to New Zealand Journal of Forest Science.

Prihatini I., Glen M., Wardlaw T., Ratkowsky D.A. & Mohammed C.2014. Needle fungi in Tasmanian Pinus radiata plantations in relation to elevation and rainfall. New Zealand Journal of Forest Science (submitted, currently under review).

II. LITERATURE REVIEW

II.1. IMPORTANCE OF *P. RADIATA*

Pinus radiata (commonly referred to as radiata pine) is a versatile fast-growing softwood tree. It has been highly developed as a forest plantation species and has attracted great interest from industry and science over a long period of time (Simberloff et al. 2010). It is the most widely planted exotic species in the world covering a total of over 4 million ha (Mead 2013). *Pinus radiata* is mainly planted in southern hemisphere including New Zealand (NZFOA 2012),

Chile (Álvarez et al. 2012), Australia (DAFF 2013), South Africa and Argentina (Simberloff et al. 2010). Chile has close to 1.5 million ha of *P. radiata* plantations (Álvarez et al. 2012) covering about 63% of the total forest plantation estate. Spain and South Africa have moderate areas of *P. radiata* plantations, followed by Italy and Argentina (Mead 2013). It is a main species of forest plantation in New Zealand and comprises 90% of this country's total forest plantations with more than 1.5 million ha (NZFOA 2012). In Australia, it comprises 75% of the 990,000 ha of softwood plantation in Australia, or 42.7 % of the total 1.74 M ha plantation estate (ABARES 2012).

Pinus radiata is a North American conifer (native to three very limited areas located in Santa Cruz, Monterey Peninsula, and San Luis Obispo Counties) and known as Monterey pine in United States of America and some other countries (McDonald and Laacke 1990). The name "radiata" was officially described in 1835 by Professor David Don from Kings College, London, UK and refers to its radiating cone scales (Mead 2013). It was initially planted as a commercial species in Victoria in 1880 after it proved the most outstanding pine species amongst several species of pine experimentally planted in Australia to assess both their commercial and aesthetic potential (Algar 2000). It is the most widely used commercial pine species in most states of Australia apart from the more sub-tropical states of Queensland and the Northern Territory. In Western Australia, South Australia, New South Wales, Victoria and Tasmania it is easy to grow, has rapid growth and produces good timber. Although some other species, such as *P. canariensis* and *P. palustris*, produce a better quality timber, their slow growth make them unprofitable (Spencer et al. 1995). *Pinus radiata* is generally managed over a 25-35 year rotation (Turner and Lambert 2013).

Pinus radiata contributes significantly to Australia's economy (ABARES 2012). It may be used for a wide range of purposes, including light construction, furniture, panelling, internal flooring, mouldings, joinery, veneers and pulpwood (Berg 2012). It is the only pine

species grown in Tasmanian plantations, covering 75,000 ha plantation estate in 2010 (Gavran 2012) and in 2008, contributed around AUD $18.5M to the Tasmanian economy in sawlog and pulpwood production of 267,680 m^3 and 243,563 m^3, respectively (Forestry Tasmania 2008).

II.2. FOREST PLANTATION HEALTH

Forest plantation health requires proper management as it influences tree growth (Beltrán et al. 2013) and poor health may cause significant economic losses. Abiotic factors such as fire, temperature, water and nutrient availability and/or biotic factors such as mammal browsers, insect and fungal pests are drivers of forest health. The threat of pests to forest plantations applies at any stage in the rotation, from planting time to established plantations near harvest. Pests, diseases and their propagules are vectored all over the world through pathways such as germplasm movement or the importation of wood and forest products (Webber 2010). As forestry plantations expand in many countries and with increased global trade and rapid movement of goods and people, the problems of pests and diseases in these resources have grown exponentially (Webber 2010).

With climate change, the impact of pests and diseases on trees is likely to change as the damage they cause to their hosts has been shown to be correlated to climatic factors such as rainfall, humidity and temperature (Tubby and Webber 2010, Elad and Pertot 2014). Several studies indicate that this will be the case with *P. radiata*. For instance, modelling has shown that change in temperature and soil moisture may potentially increase the risk of establishment of pitch canker caused by *F. circinatum* in Australasian *P. radiata* plantations (Ganley et al. 2011). The risk of pitch canker establishment is also predicted to increase as climate changes for *P. radiata* in Chile, Western Europe, and the west coast of North America

(Watt et al. 2011b). Sphaeropsis shoot blight is predicted to increase as the frequency and severity of drought increases over the next 30 years but will reduce in the following 40 years (Fabre et al. 2011). The predictions of how DNB will respond to climate change vary depending on region; the overall risk of disease incidence is predicted to decrease in the southern hemisphere but increase in Scandinavia, Eastern Europe, and British Columbia (Watt et al. 2011b). This disease is reportedly already responding to climate change in British Columbia with outbreaks occurring during wet and warm conditions (Welsh et al. 2014).

II.3. HEALTH PROBLEMS IN *P. RADIATA* PLANTATIONS

Pinus radiata plantations around the world can suffer from many damaging insect pests and fungal diseases. The insect pests considered as major problems in *P. radiata* plantations are sirex wood wasp (*Sirex noctilio*) as reported in Australia (Collett and Elms 2009) and New Zealand (Watson et al. 2008), bark beetles such as *Ips grandicollis* in Australia (Yousuf et al. 2014), *Hylastes ater* in New Zealand (Reay et al. 2010), and *Essigella californica* in Australia (May 2004) and New Zealand (Watson et al. 2008). Widespread and serious fungal diseases in *P. radiata* plantations are DNB, sphaeropsis shoot blight caused by *Sphaeropsis sapinea* (Mead 2013) and pine pitch canker caused by *F.circinatum* (Ganley et al. 2011). Other diseases are present as serious pathogens but in relatively localized regions such as *Neonectria fuckeliana* in New Zealand (Ramsfield et al. 2013), phytopthora root rot of seedlings in New Zealand (Reglinski et al. 2009) and two recently emerging new foliar *Phytophthora* diseases e.g. *P. pinifolia* associated with dying *P. radiata* in Chile (Duran et al. 2008), and *Phytophthora* taxon '*pluvialis*' associated with Red Needle Cast (RNC) in New Zealand (SCION 2013), cyclaneusma needle cast (CNC) in Australia (Choi and Simpson 1991) and New Zealand (Bulman and Gadgil 2001), *Lophodermium pinastri* (Choi and Simpson 1991) and spring needle cast (SNC) in Tasmania (Podger and Wardlaw 1990a).

Needle loss in *P. radiata* due to insect pests can be significant e.g. the losses caused by the aphid *E. californica* in Australia were estimated at approximately AUD $21M per year (May 2004). Productivity of *P. radiata* can also be dramatically reduced if needles are lost through infection by fungal pathogens; in New Zealand, DNB can halve productivity (O'Hehir and Nambiar 2010) and CNC has caused losses estimated at around NZD $38M per year (Watt et al. 2011b).

II.4. NEEDLE DISEASES OF *P. RADIATA* PLANTATIONS IN THE SOUTHERN HEMISPHERE

Most of the important fungal needle pathogens of *P. radiata* are present in the southern hemisphere. New and damaging needle diseases of *P. radiata* are emerging in the southern hemisphere associated with aerial *Phytophthora* species e.g. *P. pinifolia* in Chile (Ahumada et al. 2013) and *P. pluvialis* causing RNC in New Zealand (Williams et al. 2013). Important needle casts or blights of *P. radiata* in Australia and New Zealand are DNB, CNC, SNC and physiological needle blight (PNB) (Table 1). Although two fungal pathogens are known to cause DNB, *D. septosporum* is the only species present and causing serious damage in *P. radiata* in New Zealand (Bulman et al. 2008, Watt et al. 2009) and Australia's mainland plantation estate (Ivkovic et al. 2010). DNB has been recently and extensively reviewed by Bulman et al. (2013). This review focuses on SNC and associated needle cast fungi. Pines are the most susceptible of all coniferous species to needle cast diseases (Bednářová et al. 2013).

Table 1.Summary of major needle casts or blights of *P. radiata* plantations in Australia and New Zealand; dothistroma needle blight (DNB), cyclaneusma needle cast (CNC), physiological needle blight (PNB) and spring needle cast (SNC).

	DNB (Bulman et al. 2013)	**CNC** (Bulman and Gadgil 2001)	**PNB*** (Bulman 2002, Gould 2006)	**SNC** (Podger and Wardlaw 1990a)
Symptoms on needles	Red band in green or yellowing needles (Bradshaw 2004)	Needles become yellow/brown with degree of shedding	Red brown needle discoloration and premature death but needles remain attached	Rapid browning of needles, 1 yo needles are shed
Period	Late summer - spring (June - October)	Major in spring (September - November) Minor in autumn (March - May)	winter - spring (May -November)	spring (September - November)
Fruit body	Black conidiomata present	White ascocarps (blisters) (Figure 1)	No reproductive structure observed	Black conidiomata and white ascocarps might or might not be present**
Effect	1-15 yo	6-20 yo	> 15yo	> 7 yo starting at canopy closure

* PNB is a needle cast of unknown etiology found in New Zealand but not reported in Australia

** Several fungi have been reported in associated with SNC-affected needles (Podger and Wardlaw 1990a)

NB: this table does not list the new *Phytophthora* species *P. pluvalis*

II.4.1. SNC Disease in Tasmania

There are several serious needle casts of *P. radiata* for which the causal agent(s) are not known. PNB in New Zealand and SNC in Tasmania are such needle casts of *P. radiata* but have very different symptoms (Table 1). Spring needle cast appears in young pine plantations at canopy closure (age 6 -7 years) and is manifested by the rapid browning of one-year-old needles, the symptoms starting in early spring, followed by the heavy casting of needles (Table 1; Figure 3). It is especially common in wet areas of high altitude (Podger and Wardlaw 1990a). Spring needle cast leads to growth reductions as defoliation affects clearwood (pruned and thinned) plantations during the latter half of their rotation. Stands with moderate or severe SNC can be expected to suffer potential losses in clear-fall volume of 30-50% (Podger and Wardlaw 1990a).

The causal agent(s) of SNC in Tasmanian have not been clearly identified (Podger and Wardlaw 1990a). Spring needle cast is not considered as a classical needle blight disease caused by a primary fungal pathogen (Podger and Wardlaw 1990a). It is thought to be caused by a suite of endophytic fungi that are triggered into secondary pathogenic activity by an environmental stress (Elliot and Wardlaw 2003, Wardlaw 2008). No specific fruiting bodies have been consistently present in the needles of trees with SNC, but *Cyclaneusma minus*, *Lophodermium pinastri* and *Strasseria geniculata* have been putatively associated with SNC in Tasmania with (Podger and Wardlaw 1990a).

II.4.1.1. *Cyclaneusma minus*

The genus *Cyclaneusma* belongs to the family Rhytismataceae, order Rhytismatales, which was created by Dicosmo et al. (1983) to accommodate two species previously included in the genus *Naemacyclus*, but having ascospores and hymenium with quite different characteristics to the type species of *Naemacyclus*, *N. fimbriata (= N. pinastri)*. Two different species, i.e. *C. minus* and *C. niveum* are recognised in *Cyclaneusma* (Dicosmo et al. 1983) though it has recently been proposed that these two species revert to *Naemacyclus* (Lumbsch and Huhndorf 2010). This would result in 25 species of *Naemacyclus*, excluding those that have recently been moved into other genera such as *Marthamyces* (Johnston 2006). Many of these 25 species are known only from their type specimens, but the type species of the genus, *N. fimbriata*, is well known as a saprophyte of the cones of several pine species and has been recorded from needles of *Pinus rigida* (Vujanovic et al. 1998). A phylogenetic analysis based on 18S rDNA sequences grouped *C. minus* and *N. fimbriata* in a well-supported clade with approximately 2% sequence divergence across a 1 kbp fragment of DNA (Gernandt et al. 2001), but the sequence for *N. fimbriata* used in that study (AF203457.1) has less than 90% sequence similarity to another accession (FJ176811), purportedly from the same *C. minus* isolate (CBS 289.61) (Schoch et al. 2009). It is unclear which accession represents the correct sequence for that isolate (Dicosmo et al. 1983, Gadgil 1984, Sieber 1999) so the proposed reversion may be based on incorrect data. Many authors continue to refer to *Cyclaneusma minus* and *C. niveum* (McDougal et al. 2012, Watt et al. 2012).

Cyclaneusma minus is a pathogen of several species of *Pinus* and is commonly reported as having a latent or endophytic phase in symptomless needles (Gadgil 1984, Sieber 1999, Kowalski and Zych 2002). *Cyclaneusma minus* is more pathogenic than *C. niveum*, which is considered a weak pathogen (Kistler and Merril 1978, Petrini and Petrini 1985, Bulman and Gadgil 2001). Both of these *Cyclaneusma* species have been recorded in many

different species of *Pinus* including *P.brutia, P. contorta, P. halepensis, P. jeffreyi, P. monticola, P. mugo, P. nigra, P. pinaster, P. ponderosa, P. radiata* and *P. sylvestris* (Sieber 1999, Botella and Diez 2011, Farr and Rossman 2012). There is some evidence of tree to tree variation in susceptibility of *P. radiata* to infection by *C. minus* (Choi and Simpson 1991, Beets et al. 1997) but no reports on the possible existence of strains of the fungus varying in virulence could be found. The distribution of *C. minus* is worldwide, including Asia, Europe, e.g. Spain (Zamora et al. 2008), USA (Merrill and Wenner 1996), New Zealand (Bulman 1988), Chile (Toro and Gessel 1999), South Africa (Botes et al. 1997) and Australia (Choi and Simpson 1991).

Table 2. Peak season of *Cyclaneusma* needle cast symptoms and disease development described in three different locations and two different host trees.

Site	**USA** (Merrill and Wenner 1996)	**New Zealand** (Gadgil 1984)	**Australia** (Choi and Simpson 1991)
Host tree	***P. sylvestris***	***P. radiata***	***P. radiata***
Infection period	autumn (September - November)	winter - summer (July - November)	summer - autumn (November - April)
Sporulation	autumn - winter (October - December)	autumn - winter (April - August)	spring (September - November)
Needle-cast	autumn - winter (October - December)	spring - summer (September - December)	autumn (March - May)

Serious impacts due to *C. minus* are described from around the world. This pathogen has been reported damaging Pennsylvania Christmas tree plantations (*P. sylvestris*), (Kistler and Merril 1978, Merrill and Wenner 1996) and causes very significant losses in *P. radiata* plantations in New Zealand (Bulman 1988, Dick 1989), Spain (Magnani 1972), Chile (Luisi

et al. 1987) and also in South Africa (Botes et al. 1997). In Australia, this fungal pathogen has been reported from NSW, ACT (Stahl 1966, Choi and Simpson 1991, Crowley 2006), Victoria, Tasmania (Podger and Wardlaw 1990a) and South Australia (Pawsey 1967), although there is little specific information about the economic impact of CNC.

Symptoms of CNC (Figure 1, Table 2) include the mottled yellowing of needles of one-year and older needles, and the appearance of prominent transverse reddish-brown bands (Gadgil 1984, Choi and Simpson 1991). Needles eventually become mottled brown. The symptoms occur in the central and lower crown, although almost the whole crown can be affected in susceptible trees (Gadgil 1984). In Australia and New Zealand, CNC symptoms are rarely seen in very young radiata pine trees (< 3-years-old) (Angus Carnegie, Pers. Com., Van Der Pas et al. 1984, Bulman, 1988) although needles become susceptible to infection in the midsummer of the first growing season (Bednářová et al. 2013). Symptoms do not usually become noticeable in the field until trees are 6-years-old. The most severely affected are trees aged 6-20 years, while older stands (>25-years-old) are less severely affected although not fully resistant to the disease (Bulman 1988).

As for most fungal pathogens, *Cyclaneusma minus* infection is affected by temperature, humidity and the availability of inoculum (Merrill and Wenner 1996, Porras-Alfaro et al. 2011). Current year needles are resistant to infection until they are 8 or 9 months old (Bednářová et al. 2013) and can be infected from autumn to early winter. A study has shown that *C. minus* infection can occur between a wide range of temperatures i.e. 10°C to 25 °C (Gadgil 1984). In New Zealand, if there is a massive amount of inoculum available on the forest floor, a heavy rainfall period in autumn to winter provides adequate needle wetness for infection to take place (Porras-Alfaro et al. 2011), therefore severe needle cast events can be expected during spring after a wet and relatively mild autumn and winter (Bulman 1993). Needles begin to develop symptoms of infection by midwinter (Table 2), and are usually cast

in early spring, when they are about 1-year-old (Gadgil 1984) although this can vary between locations (Table 2). In Australia needles are more often cast in autumn (Choi and Simpson 1991).

Light-coloured fruiting bodies (ascocarps) develop on all needle surfaces after about one year of infection (Bulman 1988, Choi and Simpson 1991). Ascocarps of *C. minus* can be found on both the abaxial and adaxial sides in brown needles, still attached to the twig or on the ground, mostly during wet periods in autumn, winter and spring (Choi and Simpson 1991). The epidermis of the needle ruptures into two flaps and exposes the apothecia. Apothecia develop beneath the epidermis about one month after symptoms first appear (Merril 1980, Gadgil 1984, Choi and Simpson 1991). Apothecia are elliptical, initially waxy, 200-600 μm long, sub-hypodermal and concolorous with the dead needles, when mature. Asci are cylindrical with acutely pointed tips, and each contains eight ascospores (Chastagner 1997).

Propagules are the airborne ascospores which are discharged from apothecia on dead needles (Kistler and Merril 1978, Karadzic and Zoric 1981, Gadgil 1984). In America, *C. minus* is categorized as having a high risk of spread, e.g. capable of dispersing more than several kilometres per year through its own movement or by abiotic factors (such as wind, water or other vectors) and demonstrates an ability for redistribution through human-assisted transport over long distances (USDAFS 1993).

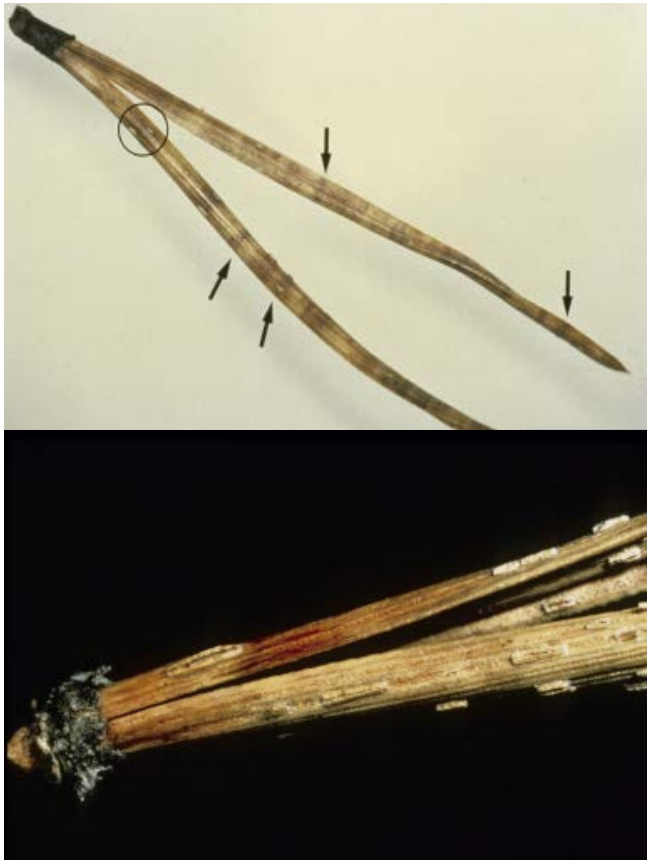


Figure 1. Symptoms of *Cyclaneusma* needle cast disease (upper) and *Cyclaneusma* ascocarps in pine needles (below), images from Joseph O'Brien, USDA Forest Service, Bugwood.org.

CNC disease, as mentioned above, is easy to identify by the observation of the symptoms on the tree, the morphology of the fruiting bodies present on the needle and fungal cultures. Variation in morphological characters of *C. minus* cultures (as *Naemacyclus minor*) was initially reported by Karadzic (1981); variation of cultural morphology between populations has also been reported (Beets et al. 1997, Bulman and Gadgil 2001). Differences in mycelium colour, colony texture, pigmentation, sporulation and growth *in vitro* have been used to separate *C. minus* into two different morphotypes i.e. *C. minus* 'verum' and *C. minus* 'simile' (Bulman and Gadgil 2001). A study of genetic variation in *C. minus* cultures from NSW, Australia and New Zealand using RAPD, SSR and IGS molecular markers revealed two main groups in *C. minus* that corresponded to grouping by morphological characters,

though high similarity of rDNA ITS sequences indicated that they were closely related (Crowley 2006). A recent phylogenetic analysis (Prihatini et al. 2014a) confirmed the existence of two separate species in *C. minus,* previously recognised as distinct cultural morphotypes, termed *C. minus* 'verum' and *C. minus* 'simile' (Dick 2001).

A study on *P. radiata* in New Zealand, found that production was reduced by up to 10.1 m^3/ha for each 10% increment in disease severity (Van Der Pas et al. 1984), while disease severity of up to 80% caused a 60% loss in average volume increment (Bulman 1988). Another study found that losses could be up to 71 m^3/ha at the end of the rotation, if half of the final crop trees suffered from the disease (Bulman 1993). In ornamental pines such as *P. sylvestris* the disease resulted in loss of aesthetic and can be rendered unprofitable (Merrill and Wenner 1996).

Applications of Chlorothalonil fungicides in seven year old *P. sylvestris* plantations in Pennysylvania were proven to decrease the needle cast caused by infection of *C. minus*, but repeated applications of fungicides and selection of genetic materials were required (Merrill and Wenner 1996). In New Zealand, the laborious method of fungicide application by injection to 8.5-years-old *P. radiata* for three years was also proven to reduce needle loss (Hood and Vanner 1984), but aerial applications of fungicides have been unsuccessful (Vanner 1986). Despite the success of systemic fungicide injection in controlling disease, this method is not feasible for large-scale plantations (Bulman and Gadgil 2001) .

Cultural practices that minimize free moisture, such as wide spacing, pruning and thinning have been applied to control CNC (Bulman and Gadgil 2001). Pruning and thinning at an early age (4-5-years-old) gives no significant reduction in disease severity while a delayed thinning starting at age 7-8 years and up to 10 years clearly reduces disease severity (Bulman and Gadgil 2001). Thinning at a later age also provides an opportunity to remove

susceptible trees (Bulman and Gadgil 2001). Weed control helps to limit the spore germination of pine needle pathogens by maintaining less humid conditions. However, the selection of resistant trees through breeding programmes is seen as the most effective way to manage needle casts (Bulman et al. 2008).

Observations of variation in susceptibility to *C. minus* have been found in different host tree species, e.g. *P. sylvestris* in the USA (Merrill and Wenner 1996). Another study revealed that the populations of *P. radiata* in New Zealand were more resistant to CNC than Californian mainland populations of *P. radiata* (Burdon et al. 1992). Study of genetic resistance to *C. minus* in *P. radiata* populations in New Zealand found that narrow-sense and family-mean heritability estimates were in the range of 0.0-0.7 (Dungey et al. 2006). The majority of narrow-sense estimates were between 0.2 and 0.3, whereas the majority of family-mean estimates were between 0.6-0.7. On sites known to be prone to CNC, heritabilities were low to moderate and genetic correlations between sites were moderate to high. It appears therefore that there are good indications that breeding for resistance to CNC will be successful, which would significantly improve forest productivity in needle cast-prone areas.

II.4.1.2. *Lophodermium pinastri*

Lophodermium is a large genus of over 100 species in the Rhytismataceae (Lantz et al. 2011). Some of these species are endophytes and pathogens of conifers (Bednářová et al. 2013, Rajala et al. 2013). The genus was reported present in a variety of conifers, such as *L. cathayae* in *Cathaya argyrophylla* (Cathay silver fir) in China (Gao et al. 2013), *L. piceae* in *Picea abies* (Rajala et al. 2014) and in *P. mariana* (Sokolski et al. 2007). Many *Lophodermium* species are found on pine but relatively few cause disease (Minter 1981). *Lophodermium macci* is reported as a pathogen in five-needled pines (Sokolski et al. 2004). *Lophodermium seditiosum* is the only known needle cast pathogen of two- and three-needled

pines (Hanso and Drenkhan 2012) in natural stands or plantations e.g. *P. sylvestris* (Stenström and Ihrmark 2005, Hanso and Drenkhan 2012). *Lophodermium* needle cast caused by *L. seditiosum* is associated with a spring needle cast that is manifested by very small yellow spots around infection sites, although these symptoms are also similar with other causal agents such as ozone, winter burn and insect attack (Bednářová et al. 2013).

Before the studies of Minter and others in the late 1970s e.g., Minter et al. (1978), many collections of *Lophodermium* on pine needles were referred uncritically as *L. pinastri*. Minter (1981) distinguished the pine-inhabiting *Lophodermium* species by morphological features such as the position at which the ascomata develop within needles, the degree to which the lower wall of the ascoma develops, the occurrence of zone lines on needles, and the presence or absence of anamorph conidiomata (Stenström and Ihrmark 2005). *Lophodermium pinastri* has thus been long confused with the pathogen *L. seditiosum* (Johnston et al. 2003, Stenström and Ihrmark 2005) but is now considered as an endophyte which fruits on old needles and is associated with decline e.g. secondary to infection by *L. seditiosum* (Hanso and Drenkhan 2012, Bednářová et al. 2013). *Lophodermium seditiosum* does not produce black diagonal lines between individual hysterothecia as does *L. pinastri* (Bednářová et al. 2013). *Lophodermium seditiosum* although present in Europe, North America and Asia, is considered a quarantine pathogen in Australia and New Zealand as it has not been identified from these countries. *Lophodermium pinastri, L. molitoris* and *L. conigenum* are among the species reported from the Australasian region on temperate pine species (Johnston et al. 2003, Simpson and Grgurinovic 2004) and *L. australe* on tropical pine (Minter 1981) but there are no reports of serious disease associated with these species.

The similarity of fruit body and spore morphology among different *Lophodermium* species means that identification based on these characters is very difficult (Stenström and

Ihrmark 2005) and exact identification is facilitated by DNA analyses (Hou et al. 2009, Lantz et al. 2011).

II.4.1.3. *Strasseria geniculata*

Strasseria geniculata (Nag Raj 1983) has many synonyms such as *Phoma geniculata* and *Allantophoma nematospora* (International Mycological Association 2014). It is generally considered as a saprobe. Mycologists in the UK have reported this species from the litter (leaves, needles, twigs) of a variety of non-woody and woody hosts including coniferous trees, such as *P. nigra, P. strobus, P. halepensis, P. sylvestris, Abies alba* and *Picea sitchensis* (British Mycological Society 2014). A herbarium specimen exists at Kew from Austria, Central Europe (Kew Royal Botanic Garden n.d.). In the southern hemisphere, *S. geniculata* has been reported from fallen needles of *P. radiata* in New Zealand (Gadgil and Dick 1999) and Tasmania (Podger and Wardlaw 1990a) although not officially reported as present in Australia (Global Biodiversity Information Facility n.d.). In Tasmania, the fruiting bodies of *Cyclaneusma, Lophodermium* and *Strasseria* have all been frequently present on needle litter under SNC affected trees, but only those of *Lophodermium* and *Strasseria* were observed (occasionally) on attached needles (Podger and Wardlaw 1990a).

In New Zealand, *S. geniculata* has been found in association with the episodic defoliation of *P. radiata* which has occurred over a number of years in Northland, East Cape, Westland and the central North Island (Bulman 2002). Symptoms of the "*Strasseria*" defoliation appear during late winter and spring when foliage on affected trees becomes red-brown and then turns khaki. The needles droop but remain firmly attached to branches after death and may still be present on the tree in late November or December. Distribution within a stand is usually localized and occasionally symptoms are present on nearly every tree. CNC differs in that the distribution of the disease is usually scattered within a stand, needles turn

firstly yellow and then brown, the needles tend not to droop but detach very readily, most being cast by late October. It is not known whether *S. geniculata* plays a role in needle death in the southern hemisphere needle casts of SNC in Tasmania or "*Strasseria*" defoliation in New Zealand or merely take advantage of senescent or dead material as opportunists. The needle cast has been referred to as "*Strasseria*" in New Zealand for convenience but no causal association has been proved or is implied.

II.4.4. Environmental triggers of SNC

Podger and Wardlaw (1990a) considered representations of the climatic envelopes for *P. radiata* and that SNC could be related to mean annual rainfall and mean annual temperature, concluding that it is clearly related to cool humid environments. Wardlaw (personal communication) hypothesizes that on high altitude wet sites in Tasmania, a potential source of environmental stress could be caused by the lag between the increase in air and soil temperatures during late winter and early spring. Thus while increasing air temperature triggers shoot activity, low soil temperature and therefore low root activity means that the demand for nutrients and/or water exceeds their supply, potentially causing stress. This putative environmental stress could trigger the secondary activity of endophytic fungi and the expression of SNC in young trees of *P. radiata*. Spring needle cast is manifest at canopy closure when trees are 6-7-years-old, which would also favour the development of needle fungi.

II.4.5. Management of SNC

Several management strategies for SNC have been attempted (Podger and Wardlaw 1990b). Thinning and pruning prior to onset of SNC (between age 5 and 8 years) (Podger and Wardlaw 1990b) did not significantly reduce the SNC impact as the disease symptoms were

expressed 2-5 years after thinning or pruning (Wardlaw 1994). The experimental applications of chlorothalinol at six-weekly intervals in a 7-year-old stand showed an increased retention of needles compared to treatments with other fungicides (Podger and Wardlaw 1990b). However, it is not economically or environmentally feasible to spray fungicides in *P. radiata* plantations as annual applications from age 12 to approximately age 25 years would be required (Wardlaw 1994).

Figure 2. *Pinus radiata* tree with severe SNC next to a healthy tree at the Oonah marker-aided selection trial in Tasmania (top). Needles affected by SNC (bottom); with white ascocarps of *Cyclaneusma* (left) and black conidiomata of *Lophodermium* (right).

Many studies have explored the exploitation of genetic resistance to pest and diseases for commercially important timber species such as *Pinus* (Liu and Ekramoddoullah 2011, Iturritxa et al. 2013). However, silvicultural selection for desirable wood traits may not coincide with selection for resistance, in part because of a reduction of chemical and genetic diversity (Carraway 2001). Selection of trees for resistance to insect pests and pathogens requires an assessment of trial plantations for heritable variation in levels of damage. Identifying rare resistant genotypes using markers may be difficult because many factors interact to produce a resistant phenotype (Henery 2011). Despite the unknown etiology of SNC, tolerance to SNC has been investigated especially in light of the observation that the severity of SNC can vary significantly among trees in the same plantation; in 1988 in a clonal seed orchard at Upper Natone (North-West Tasmania) planted in 1973 (Podger and Wardlaw

1990b) and in four field trials established in 1986 North-West of Tasmania (Podger and Wardlaw 1990b). These two studies showed that resistance to SNC is correlated with host genetics and is moderately heritable with values of 0.253 and 0.37 (Podger and Wardlaw 1990b, Kube and Wardlaw 2002b).

In 1989, a third SNC assessment was conducted in a progeny trial that involved over 300 Australian and New Zealand families at Upper Castra in Tasmania. The study found that seven crosses from Australia could be categorized as resistant to SNC and 14 crosses from New Zealand as highly resistant (Wardlaw 1994). This assessment confirmed that host genetics played a role in the response to this disease. The breeding values of this trial were calculated and trees selected for a Marker Aided Selection trial (Kube and Piesse 1999). Marker aided selection (MAS) is a process whereby a marker (morphological, biochemical or one based on DNA/RNA variation) is used for the indirect selection of a genetic determinant or determinants of a trait of interest (i.e. productivity, disease resistance, abiotic stress tolerance, and/or quality). The MAS trial was established in 1999 at Oonah, Tasmania, to provide materials for breeding *P. radiata* resistant to SNC (Kube and Piesse 1999).

II.5. ENDOPHYTES AND FOREST HEALTH

The most used definition of an endophyte is '*All organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to the host*' Petrini (1991). The endophyte-host relationship can best be described as a '*balanced symbiotic continuum ranging from mutualism through commensalism to parasitism'* (Aly et al. 2011), but it may become pathogenic (Rodriguez and Redman 2008). Sieber (2007) states that these '*pathogens*' must at some time sporulate, either when leaves senesce, or when the plants are stressed or produce fruit that eventually rots.

Endophyte-host plant interactions are poorly understood but are influenced by the mode of transmission, pattern of infection, plant age, environmental conditions and genetic background (Arnold et al. 2003, Aly et al. 2011). Vertically transmitted endophytes (systemic through seed) are less likely to be antagonistic at some stage in the life cycle than horizontally transmitted endophytes (non-systemic via spores) (Saikkonen et al. 1998). Most of the switches from a mutualistic to parasitic interaction are triggered by stress (e.g. host nutrient imbalance) (Aly et al. 2011).

Endophytes may provide the plant with various ecological advantages. They may increase the nutritional content of their host plant, increase resistance to drought and water stress, increase tolerance to high temperature, salinity, and high metal content (Aly et al. 2011). These ecological advantages may be mediated through the stimulation of host growth and/or secondary compounds (Bultman et al. 2004) such as antioxidants e.g. flavonoids and other phenolic antioxidants (Aly et al. 2011). Many natural products that are known to confer protection against biotic stress are produced by endophytic organisms. Several fungal endophytes produce toxic alkaloids which defend grasses against the herbivory of ungulates and insects (Belesky and Bacon 2009) and some endophytes protect their hosts by entomopathogenic behaviour - they colonize the exoskeleton of living insects and finally kill them (Jaramillo et al. 2009). Endophytes are being increasingly recognized and manipulated for their role in protecting their host plants from natural enemies such as insects (Jaramillo et al. 2009), nematodes (zum Felde et al. 2009) and pathogenic fungi (Ownley et al. 2010).

Several reviews of endophytes in woody plants have been published (Moricca and Ragazzi 2007, Albrectsen et al. 2010, Gazis and Chaverri 2010). Endophytes have been studied from a wide variety of woody plant tissues (roots, stems, leaves) of species such as oaks (Moricca and Ragazzi 2007), tropical forest trees (Arnold et al. 2003) and conifers (Arnold et al. 2007, Kowalski and Drozynska 2011). In conifers, fungal diversity studies have

been conducted in several species as presented in Table 3, including *P. radiata*. In-depth investigations of fungal diversity have been carried out in Spain with several species of pine: *P. halepensis, P. nigra, P. pinaster, P. sylvestris, P. uncinata* (Zamora et al. 2008, Botella et al. 2010, Botella and Diez 2011).

Table 3. List of fungal diversity studies conducted in conifer.

Host tree species	Location	Author
Picea abies	southern Finland	Muller and Hallaksela (1998, 2000)
Pinus densiflora	Japan	Hata et al. (1998)
P. elliotii	Uruguay	Alonso et al. (2011)
P. monticola	USA	Ganley (2004a)
P. mugo	Switzerland and Germany	Sieber (1999)
P. nigra	Poland	Kowalski and Zych (2002)
P. radiata	South Africa	Botes et al. (1997)
	New Zealand	Ganley (2008)
P. sylvestris	Western Alps of Europe	Giordano et al. (2009)
	Northern Spain	Zamora et al. (2008)
P. tabulaeformis	China	Guo et al. (2003, 2008)
P. taeda	Northern Carolina, USA	Arnold et al. (2007)
	Uruguay	Alonso et al. (2011)
P. thunberghii	Japan	Hata et al. (1998)
	Korea	Min et al. (2014)
P. wallichiana	Western Himalayas	Qadri et al. (2014)
P. uncinata	Northern Spain	Zamora et al. (2008)

Some tree species may host hundreds of endophyte species in one tissue type but most endophytic communities studied so far are dominated by a few host-specific species (Sieber 2007, Vega et al. 2010, Botella and Diez 2011, Gonzalez and Tello 2011). Endophyte fungal communities in host species of the same plant family were found to be dominated by closely related endophyte species and as relatedness of the host plants decreases so does the relatedness of dominant endophytes (Sieber 2007). Therefore it appears that endophytic fungal communities may show single host specificity at the plant species level. Endophytic fungi may also exhibit organ and tissue specificity (Kumar and Hyde 2004).

Understanding endophyte diversity is crucial to determining the role of endophytes in forest health. Many pathogens, including serious pathogens of conifers, such as *D. septosporum* in *P. radiata* (Choi and Simpson 1991, Bradshaw 2004), *Sphaeropsis sapinea* in *P. nigra* and *P. sylvestris* (Flowers et al. 2003) and *Phaeocryptopus gaeumannii* in *Pseudotsuga menziesii* (Morales et al. 2012) have a latent phase often considered as an endophytic stage (Sieber 2007). There have only been a limited number of investigations of needle fungal endophyte communities in *P. radiata*. Studies of *P. radiata* needle fungi in the southern hemisphere have focused on the study of single pathogens such as the distributions of pathogen *Sphaeropsis pinea* and *Diplodia scrobiculata* in South Africa (Burgess et al. 2004) and endophytes that offer the promise of biocontrol, for example the characterization of endophytic *Beauveria* as biological control agents against insect pests of pine in New Zealand (Reay et al. 2010).

II.6. DETECTION AND IDENTIFICATION OF NEEDLE FUNGI

Accurate pathogen identification is important in biosecurity to help prevent the accidental introduction and rapid spread of disease agents around the world. . For example, over the last decade *Puccinia psidii,* a serious rust pathogen of myrtaceous host species, was introduced into Hawaii and then Australia, the home to half of the world's myrtaceous species (Glen et al. 2007, Pegg et al. 2013). Disease agents may be moved to new locations through many different pathways especially by travelling with their host trees or associated germplasm, e.g. *Mycosphaerella* spp. in eucalypt plantations (Coutinho et al. 1998) and *Sphaeropsis pinea* in *P. radiata* (Burgess et al. 2004). Tree species may be exposed to new pathogens when grown in new locations (Barber et al. 2008, De Blas et al. 2009, Kawanishi et al. 2009). Unambiguous identification of pathogens is also important for appropriate management of disease, as different diseases respond to different control strategies.

Several methods are used to detect and identify fungi (including endophytic fungi in plant tissue). These include histological observation, staining the host tissue and examining it under a microscope to observe fungal structures within or on the host tissue (Johnston 2006). Cultivation-based identification, involves surface sterilization of the host tissue, plating the fragment onto growth media followed by morphotype observation and DNA identification (Deckert and Peterson 2000, Stefani and Berube 2006, Collado et al. 2007). Cultivation independent identification is based on immunological methods (Aegerter and Gordon 2006) and direct amplification of fungal DNA from colonized plant tissues (Gao et al. 2005, Arnold 2007).

A limitation of histological observation is its sensitivity, especially for endophytic colonization which is often localized and recognition of small fungal structures is not easy in certain plant tissues (Schulz and Boyle 2005) especially pine needle tissue. Cultivation dependent methodology for fungal identification has been common including for the study of endophytes (Unterseher and Schnittler 2009). Surface sterilization methods (Schulz and Boyle 2005) must be established prior to isolation processes in order to optimize the yield of many fungi (especially endophytic fungi) and to remove contaminating epiphytic fungi. Different types of host material require varying periods and strengths of sterilization compounds (Hyde and Soytong 2008). Morphotaxa, based on gross colony features, are used frequently as functional or operational taxonomic units (OTU) (Arnold et al. 2003), although many endophytic cultures do not produce fruiting structure and lack the morphological characters needed for accurate species identification.

The main consideration with cultural studies especially of endophytes is that the fungi isolated in any study are only those that grow out (Gao et al. 2005, Sun and Guo 2012). Some fungi including endophytes are unable to grow in culture, grow very slowly or require specific media. The faster-growing culturable fungi are those obtained by traditional methodology and

it is highly probable that some or even numerous endophytes are never isolated (Hyde and Soytong 2008). A method of bacterial high-throughput culturing (HTC) based on techniques of ‘dilution to extinction’ or ‘extinction culturing’ has been adapted to endophyte fungi to generate a larger number of cultures (Unterseher and Schnittler 2009). The low-density partitioning of colony forming units in tubes or micro-wells exploits the fact that the culturable species diversity increases as inoculum density decreases (Collado et al. 2007).

Cultivation independent molecular methods permit the detection and identification of fungi and are especially relevant for those fungi that are not easily culturable (Zuccaro et al. 2003, Unterseher and Schnittler 2009). A paucity of distinguishing morphological characters and/or the failure to produce the recognisable sexual stage in culture can also be an obstacle to the identification of species and generic relationships among fungi. DNA sequences provide an abundance of characters for phylogenetic analysis and have been frequently used for this purpose in the last three decades. Total DNA can be isolated from plant tissue and directly amplified with fungal primers. Denaturing gradient gel electrophoresis (DGGE) or cloning of PCR products may then be used to separate the fragments of DNA (Arnold 2007); subsequent sequencing and phylogenetic analysis theoretically enables the discrimination of the fungal species colonizing a portion of plant tissue.

Studies frequently use direct DNA analysis from plant tissue e.g. grasses (Porras-Alfaro et al. 2011, Herrera et al. 2013) or wood (Premalatha and Kalra 2013) to characterize fungal communities. The use of fungal-specific primers in the PCR prevents amplification of DNA from the host (Bruns et al. 1991). Most studies of endophytic fungi in pine needles have relied on isolated fungi from surface-sterilized needles e.g. Gourbiere and Debouzie (2013) and Ganley (2006).

DNA sequence analysis is increasingly being used for identification of fungal cultures and for detection of species in the absence of fungal isolation (Collett and Elms 2009, Sun and Guo 2012). It is essential for discrimination of OTUs in studies that do not rely solely upon culturing for identification of those OTUs. Increasingly, ecological studies are making use of metagenomic data directly from environmental samples and therefore relying completely on DNA barcodes for species identification (Van Elsas and Boersma 2011). Identification is based upon sequence similarity between the unknown isolate (or clone or sequence) and a database of reference sequences from identified isolates or herbarium specimens. This database may be derived from a privately-owned collection of specimens, where responsibility for sequence quality and correct identification of reference sequences rests with the collection owner, or from a range of publicly available DNA sequence databases, such as GenBank, EMBL and DDBJ. In the latter case, responsibility for sequence quality and correct identification lies with the submitter of the sequence data and it is often not possible for a third-party user to verify the sequence quality or that the identification is correct (Nilsson et al. 2006).

Increased confidence can be obtained where a publication or online database provides additional details. A range of initiatives based upon a barcode concept are attempting to address this problem by establishing publicly available DNA databases that require submission of sequence chromatograms to verify sequence read quality, and voucher specimens to permit morphological re-examination of specimens whose identity may be in doubt (Lombard 2011). Such databases are usually targeted to a particular group of species that may be a functional group e.g. UNITE (Browser 2009), qBOL (Bonants 2012) or a taxonomic group (e.g. FusariumID, (Geiser et al. 2004) and are necessarily more limited than GenBank and its ilk.

II.6.1. DNA barcodes for fungal identification

The concept of DNA barcoding was developed to assist in the identification of life-forms which lack the full suite of morphological characters on which species were originally described e.g. insect larvae, fungal isolates, body parts of fish and mammals (Haliassos et al. 2001, Gressel and Ehrlich 2002, Hebert et al. 2003) and plants (Kress et al. 2005). A DNA barcode consists of a short fragment of DNA sequence that has low intra-specific variability and higher inter-specific variability, allowing identification to species level on the basis of sequence similarity (Hebert et al. 2003). Two requirements for DNA barcode submissions greatly improve the reliability of identifications based on these compared to sequences from the international nucleotide sequence databases (INSDs), e.g. GenBank, the European Nucleotide Archive and the DNA Databank of Japan. The first is that sequences deposited in the barcode databases must be backed up by vouchered specimens in public culture collections or herbaria to allow independent verification of species identity. The second requirement is the inclusion in the database of sequence chromatograms, facilitating independent verification of sequence data.

International barcode databases have been developed to facilitate identification of many animals, including invertebrates, fish and birds (Hebert et al. 2003, Hebert et al. 2004) and plants (de Vere et al. 2012). Although some specialised sequence databases for fungal identification exist (e.g. UNITE for ectomycorrhizal fungi, FusariumID for *Fusarium* spp. (Kõljalg et al. 2000, Geiser et al. 2004), establishment of a universal fungal barcode database has been delayed by the lack of one or two gene regions that adequately discriminate species in all fungal groups and disagreement over which regions to use. Consequently the fungal barcode database does not yet provide a probability of placement to a taxon (BOLD_Systems 2012). Recently, the rDNA ITS was accepted as the primary barcode region for fungi, though it was recognised that resolution of species in some genera may require additional loci

(Schoch et al. 2012). *Mycosphaerella* (Quaedvlieg et al. 2012). *Fusarium* (Geiser et al. 2004, Zhao et al. 2011), *Colletotrichum* (Weir et al. 2012) and *Ceratocystis* (Harrington et al. 2014) are four genera in the Ascomycota for which the rDNA ITS is inadequate to discriminate species and require the use of loci such as translation elongation factor 1, RNA polymerase II sub-unit 1 (Geiser et al. 2004) or Beta-tubulin (Zhao et al. 2011) alone or in addition to the ITS (Quaedvlieg et al. 2012). The rDNA ITS has proven suitable for species discrimination in many genera of Basidiomycota (Seifert 2009) with the vast majority of interspecific comparisons having greater than 1% sequence variation and the vast majority of intraspecific comparisons varying by less than 1% (Osmundson et al. 2013). Exceptions include *Gymnopilus* (Rees and Ye 1999) and the *Mycena pura* species complex (Harder et al. 2013). No alternative candidate loci have been proposed for *Gymnopilus*, but translation elongation factor sequences effectively separated the phylogenetic species within the *M. pura* complex. Universal primers for these alternative regions are not available and corresponding sequences have a much lower representation in INSDs (Seifert 2009).

Several gene regions have been used to study fungi, including the rDNA ITS (White et al. 1990, Bruns and Gardes 1993), mitochondrial LSU (Blankenship et al. 2001), Elongation factor (Carbone and Kohn 1999), Cytochrome oxidase (Bruns 1999) and Beta-tubulin (Glass and Donaldson 1995). The selection of a gene region for fungal barcodes has provoked world-wide discussion (Begerow et al. 2010). The mitochondrial cytochrome oxygenase I (COX1) gene was initially proposed to be applicable to all life forms (Gould et al. 2009), but this region is unsuitable for many fungi (Gilmore et al. 2009, Dentinger et al. 2011). Eventually the ribosomal DNA internal transcribed spacer regions (rDNA ITS) were selected as the most informative barcode region across a wide range of fungal species (Bellemain et al. 2010, Schoch et al. 2012), even though additional gene regions will be required to separate species in particular genera, including economically significant genera such as *Fusarium* (O'Donnell

et al. 2010). Factors influencing this choice included the already broad acceptance of this region and demonstration of its utility in phylogenetic analyses and species-level discrimination for a wide range of taxa and the easy accessibility by PCR amplification using primers targeting the conserved flanking regions. Relying solely on a given level of sequence similarity to discriminate species may result in splitting of some species, and lumping of others. The use of phylogenetic analyses has been proposed as a more robust method for species identification (Begerow et al. 2010) that does not depend on a previously determined cut-off level.

II.6.2. Phylogenetic analysis

Phylogenetic analysis is a means of inferring evolutionary relationships, principally among species (Binder et al. 2013), but can also be applied to populations within a species (Jimenez-Gasco et al. 2014) or genes across species (Sillo et al. 2013, Yin et al. 2014). It usually involves construction of an evolutionary tree, which can be subjected to a range of statistical tests, depending on the tree-building method. The dataset consists of a matrix of taxa by characters that may be morphological (Zhang and Minter 1989, Benny and Benjamin 1991), biochemical (Stasz et al. 1989), molecular (Zhai et al. 2014) or a combination of types (Witthuhn et al. 2000). In this review the focus is on molecular phylogenetic analysis, though most methods are equally applicable to other datasets. Molecular phylogenetic analyses have risen in popularity with the increase in DNA sequence availability. In molecular phylogenies the evolutionary trees are constructed using a matrix of DNA or protein sequences where each nucleotide or amino acid is a distinct character (Baldauf 2003).

Phylogenetic studies are particularly useful in resolving taxonomic questions, e.g as a complement to morphological characters in identifying new species (Matsuzawa et al. 2012) or to resolve problems in species identification and to clearly delineate species boundaries

when morphological characters are insufficient (Reece et al. 2008). DNA sequences provide a huge and ever-growing resource as new sequencing technologies reduce the cost per base (Wilkening et al. 2013).

Phylogenetic trees consist of branches and nodes that reflecting the grouping of species or operational taxonomic units and the root in the base of the phylogenetic tree that implies the order of branching in the rest of the tree (Baldauf 2003) and may be likened to a 'family tree'. The first step in tree construction is selection and/or generation of the dataset, followed by alignment of the sequences from the different samples then tree construction using one (or more) of several tree-building algorithms.

Selection of appropriate ingroup and outgroup taxa is important for obtaining the best results in a phylogenetic analysis (Lecointre et al. 1993, Ware et al. 2008). In selecting an outgroup, best results are obtained by using a 'sister' species, but this may be difficult to determine or to execute e.g. *Cyclaneusma* (Prihatini et al. 2014a). Selection of a DNA region or regions will depend on the question being asked and the group under consideration, with the possibility of selecting regions that evolve faster or more slowly, depending on the resolution required. Fast-changing regions such as introns are suitable for studying relationships among recently-separated species (Basiewicz et al. 2012, Nyati et al. 2013), whereas highly conserved regions such as ribosomal DNA are appropriate for inferring relationships among phyla (Krueger et al. 2012) or even more distantly related groups such as animals, plants and fungi (Veuthey and Bittar 1998).

Increasingly, phylogenetic studies are based on multiple gene regions to increase confidence that the reconstructed tree represents a species phylogeny rather than a gene phylogeny (Cortinas et al. 2006, Douhan et al. 2008), unless of course, the study is concerned with evolution of genes rather than species (e.g. Sillo et al. (2013). The risk of creating

confusion rather than resolving it by over-reliance on a single gene region is exemplified by *Ceratocystis fimbriata*. Several new species, pathogenic to hardwood trees in SE Asia, have been delineated, largely on the basis of sequence variation in the rDNA ITS region (van Wyk et al. 2007, Tarigan et al. 2010, van Wyk et al. 2011, van Wyk et al. 2012). Even though multigene phylogenies were generated, the high variation in the ITS region overwhelmed any signal from the other gene regions (Harrington et al. 2014). Subsequent work demonstrated that intragenomic ITS sequence variation was high, with a single isolate having ITS sequence variants identical to two of the proposed new species (Harrington et al. 2014). Furthermore, isolates from several of these proposed species were interfertile (Harrington et al. 2014), refuting the hypothesis that they belong to distinct species.

A study conducted to calculate the minimum number of genes that would provide a reliable species phylogeny for yeast species concluded that phylogenies based on one or a few genes were highly likely to support contrasting topologies, depending on the selected regions (Rokas and Carroll 2005). A phylogeny based on analysis of 20 genes provided the same degree of robustness as one based on 106 genes. Despite the increase in genome-wide phylogenetic analyses (Rokas and Carroll 2005), most multigene phylogenetic analyses of fungi are based on up to five gene regions (Boehm et al. 2009, Crous et al. 2009, Taskın et al. 2010).

There are three schools of thought on the most appropriate way to combine multiple data sets, whether they be DNA sequences from multiple gene regions, or different types of data such as morphological, biochemical, molecular, etc. (Kluge 1998) advocated the ‘total evidence’ approach, i.e to combine all the available data and analyse together. This approach works well for complementary datasets that each provide support at different levels of the tree and so produces a tree with greater overall support. Others (e.g. Miyamoto and Fitch (1995)) maintain that datasets should be analysed separately and results compared by producing a

consensus tree. This approach has been adopted as a genealogical concordance phylogenetic species recognition (GCPSR) concept and has some strong proponents in mycological circles (Taylor et al. 2000, Vialle et al. 2013).

The third approach, followed by the majority of researchers, is to conduct tests for congruence (Bull et al. 1993) before combining datasets. The major difference among the three approaches is related to the 'de facto' weighting of characters, e.g. given one dataset (morphological or molecular) with 30 informative characters and another with 200 informative characters, the GCPSR approach results in a > 6x weighting of each character in the first dataset. Proponents of GCPSR argue that a single gene rather than a single nucleotide should be treated as a single character (Taylor et al. 2000, Vialle et al. 2013).

Tree-building methods fall into one of two main classes – those that use an algorithm to build a tree and those that use an optimality criterion to select the tree that best fits the data. Algorithmic approaches include cluster analysis (UPGMA) and neighbour joining. These are phenetic methods that rely on overall similarity and are unsuitable for examining evolutionary relationships as they do not distinguish between plesiomorphies and apomorphies (Saitou and Nei 1987). Trees are built from a pairwise distance matrix, starting with the two most similar taxa then adding the next closest and the next closest and so on (Saitou and Nei 1987). An additional disadvantage of cluster analysis is the assumption of a molecular clock that imposes equal branch lengths on different parts of the tree.

In contrast, Maximum Parsimony (MP) and Maximum Likelihood (ML) methods evaluate trees based on certain optimality criteria. Searching all possible tree topologies can be very time-consuming, e.g. for ten taxa there are more than 34 million possible trees while for 11 there are more than 654 million (Felsenstein 1978). Consequently most software examines only a subset of all possible trees, and procedures such as branch-swapping are

performed to maximise the probability of selecting a tree that most closely resembles the true tree (Morrison 2007).

Maximum parsimony aims to select the tree with the lowest number of stepwise changes. Often several trees with the same minimum number of steps may be produced, and a consensus tree may be required to summarise the information in all of the MP trees. This is particularly so where more than one sample is included for each species (Coetzee et al. 2001), in which case, alternative trees may simply reflect different branching orders within a species. These trees may be not significantly different in terms of species relationships and a consensus tree is likely to produce a polytomy for each species with more than 2 representatives (Simmons and Freudenstein 2011). In other cases, several equally MP trees may reflect uncertainty as to the exact branching order and relationships among species. This uncertainty may arise because of a paucity of characters with the desired resolution or missing taxa, including lineages that may have died out.

Most analyses include a measure of bootstrap support for branches in the MP tree. This involves the generation of multiple datasets by repeated sampling of the full dataset, analysis of these bootstrap datasets and production of a consensus tree (Felsenstein 1985). Support for each branch is given as the percentage of trees that include the particular group, less than 50% is not considered significant (Felsenstein 1985). Other indices that are used to assist in evaluation of the MP tree include the consistency index (CI) and retention index (RI), which describe the degree of homoplasy in the tree (Farris 1989), and the decay index, which measures the additional steps needed to lose a branch (Bremer 1988). The topology dependent permutation tail probability test (T-PTP) tests the monophyly of particular groups by comparing the lengths of trees with the group monophyletic vs. non-monophyletic (Faith 1985).

The PHYLIP package (Felsenstein) includes DNApars for MP analysis, SEQBOOT for generating bootstrap datasets and CONSENSE to produce a consensus tree. The most popular software for deriving MP trees is undoubtedly PAUP, which includes calculations of consistency index (CI) and retention index (RI). The consistency index may indicate a sub-optimal tree, or may simply represent a high level of convergent evolution in the group being examined (e.g. Diogo 2007).

Maximum Likelihood methods (Felsenstein 1981) evaluate trees in terms of the probability that the hypothetical history described by the tree would result in the observed data under a particular evolutionary model. ML methods require the designation of a model for character state changes, of which several have been proposed for DNA sequences (Yang 1994, Posada and Buckley 2004) but may be difficult to derive for morphological data. The model may be fully defined or contain elements that are calculated from the dataset (Nylander et al. 2004).

Bayesian analysis is a further refinement of ML methods that has risen in popularity recently. This approach was applied to phylogenetic analysis in the late 1970's (Harper 1978) but was not widely used until after the application of Monte Carlo Markov Chain techniques to tree perturbation (Mau and Newton 1997). The free availability of MrBayes (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) combined with the comprehensive tutorial material that is available online has no doubt contributed to the popularity of Bayesian analysis but other advantages include: 1. Bayesian analysis accommodates uncertainty in the phylogeny, in contrast to MP and ML which assume that the correct phylogeny is known (Huelsenbeck et al. 2000); 2. It is computationally more efficient than other likelihood methods, permitting the analysis of much larger trees (Yang 1994); 3. It is not necessary to choose the evolutionary model as the model that best fits the data can be selected by Bayesian methods (Ronquist and Huelsenbeck 2003).

Phylogenetic analysis can be applied to many questions in mycology and forest health, including assisting in species delimitation (Prihatini et al. 2014b) and identification of fungal samples for which morphological data are lacking (Prihatini et al submitted, Southern Forests paper). Phylogenetic analyses are frequently included to support new species descriptions (e.g Shen et al. (2014). Phylogenetic analyses may be of particular benefit in defining asexual fungal species, especially where incomplete lineage sorting has occurred (Stewart et al. 2014).

Multigene phylogenetic analysis of *D. septosporum*, the cause of red-band needle blight in *P. radiata* identified two distinct species associated with the disease, *D. pini* and *D. septosporum*, that were formerly classed as a single species (Barnes et al. 2004). Multigene phylogenetic studies have also been used to discriminate *Coniothyrium zuluense* from South Africa and *C. gauchensis* from Argentina and Uruguay, two distinct species that produce identical stem canker symptoms in *Eucalyptus* (Cortinas et al. 2006). The species distinction is significant for biosecurity in both countries. A multigene phylogeny has resolved the species and ecological diversity within the *Cladosporium* complex (Bensch et al. 2010). A similar study has also provided compelling evidence that the *Mycosphaerellaceae* and *Teratosphaeriaceae* represent numerous genera as well as the finding of several novel species by the same study (Cheewangkoon et al. 2009).

Broader taxonomic questions have also been addressed, e.g. a Bayesian approach was applied to a higher level phylogenetic classification of fungi (Hibbett et al. 2007) and a new phylum, the Glomeromycota, was proposed on the basis of parsimony and neighbour-joining analyses of small sub-unit rDNA (Schussler et al. 2001). The closest living relative of the fungi was determined to be animals, and vice versa, by MP analysis of 4 protein sequences (Baldauf 2003).

III. DIVERSITY AND IDENTIFICATION OF FUNGI ASSOCIATED WITH NEEDLES OF *PINUS RADIATA* IN TASMANIA

Abstract

Needle fungi have been extensively studied in conifers but rarely in *Pinus radiata*. Previous studies in *P. radiata* have been mainly based on fungal isolation. This work was a component of a study examining factors linked to spring needle cast (SNC) in Tasmanian *P. radiata* plantations and aimed to identify as many as possible of the fungal species commonly associated with *P. radiata* needles in Tasmanian plantations. Needle samples were collected from 13 sites representative of the range of sites in which *P. radiata* is grown in Tasmania, and fungi were detected by a direct PCR approach, and identified using barcode sequences from a reference collection of isolates from Tasmania, mainland Australia and New Zealand as well as sequences from the international nucleotide sequence databases (INSDs). The total number of molecular operational taxonomic units (MOTUs) was 153, with 128 detected by direct PCR and sequencing and only 35 operational taxonomic units (OTUs) isolated. Dothideomycetes was the most diverse group detected in this study, with many molecular operational taxonomic units (MOTUs) detected by direct PCR and not isolated. Leotiomycetes was the second most diverse order and Sordariomycetes third, with several operational taxonomic units (OTUs) being frequently isolated but rarely or not at all detected by direct PCR. DNA sequence data facilitated the discrimination and identification of OTUs, but some effort was required to eliminate sequences from poorly identified isolates in public DNA databases.

III.1. INTRODUCTION

Pinus radiata is grown extensively throughout the world and is the major softwood plantation species in Australia (Mead 2013). Tree productivity depends on needle/leaf duration (Rubilar et al. 2013) and the productivity of radiata pine plantations is compromised by needle cast diseases which cause premature needle loss (Watt et al. 2012). Some needle diseases are well understood with a known causal agent, e.g. *Dothistroma* needle blight (Bulman et al. 2013,

Watt, Ganley, et al. 2011). The causal agents of other needle cast syndromes may be more nebulous (Bulman et al. 2008, Podger and Wardlaw 1990). Koch's postulates are difficult to test with some needle-cast pathogens as these may have a latent or endophytic phase and pathogenic activity is triggered in response to an external stress, which may not be well understood (Sieber et al. 1999).

Fungal endophytes, defined as organisms that inhabit plant organs or colonize internal plant tissues without causing apparent harm to the host (Petrini 1991), may be significant modifiers of plant responses or contribute to acquired resistance (Ganley et al. 2008, Rodriguez et al. 2009). Understanding endophyte diversity is a crucial first step to determining the implications of endophytes for forests (Doty 2011, Krabel et al. 2013, Sieber 2007). Studying the complete fungal community associated with healthy and diseased needles may assist in understanding needle cast diseases by identifying those fungi that have a strong association with diseased or healthy trees. Recent advances in elucidating host-endophyte interactions have been facilitated by the use of molecular methods, both to assist in identification of fungal endophytes and to elucidate the biochemical pathways affected by these interactions (Woodward et al. 2012).

Several reviews of endophytes in woody plants have been published (Petrini 1991, Sieber 2007). Despite the existence of some cosmopolitan and almost ubiquitous fungal endophytes (e.g. Suryanarayanan et al. 2004), most endophytic communities studied so far are dominated by a few fungal species restricted to a single host species or genus (Botella and Diez 2011, Gonzalez and Tello 2011, Sieber 2007, Vega et al. 2010). Fungal communities in host species of the same plant family have been found to be dominated by closely related endophyte species and as relatedness of the host plants decreases so does the relatedness of dominant endophytes (Sieber 2007). Endophytes have been studied from a wide variety of plant tissues, including roots, stems and leaves, of coniferous species such as *Picea abies*

(Muller and Hallaksela 2000), *Pinus mugo* (Sieber, et al. 1999), *P. sylvestris* (Giordano et al. 2009), *P. monticola* (Ganley 2004), *P. nigra* (Kowalski and Zych 2002), *P. tabulaeformis* in China (Guo et al. 2008), *P. taeda* in northern Carolina, USA (Arnold et al. 2007), *P. thunberghii* and *P. densiflora* in Japan (Hata et al. 1998). A study of four *Pinus* species, *P. nigra*, *P. pinaster*, *P. sylvestris*, *P. uncinata*, in Spain showed that more endophytic fungal species were isolated from dried or fallen needles than from attached needles and more were isolated in autumn than in other seasons, though the different *Pinus* species supported similar numbers of fungal species (Botella and Diez 2011, Botella et al. 2010, Zamora et al. 2008).

There have been few studies on fungi in *P. radiata* needles and most of them have targeted specific types of endophytes or pathogens in latent phases (Burgess et al. 2004, Reay et al. 2010). Competition between specific pathogens and endophytes has also been studied, e.g *Dothistroma pini* may obtain an advantage over other pine-needle inhabitants, such as the latent pathogen *Cyclaneusma minus* and the endophyte *Lophodermium conigenum* by toxin production (Bradshaw 2004). An investigation into the distribution of endophytes in South Africa showed a consistency in the dominant fungi present in *P. radiata* plantations but the numbers of fungal isolates were highly variable between seasons (Botes et al. 1997). A study of radiata pine needle endophytes in New Zealand revealed significant differences in endophytic mycota between trees resistant or susceptible to needle cast diseases including *Cyclaneusma* needle cast and Physiological Needle Blight (Ganley 2008). Differences observed included both the species present and the number of species detected. *Cyclaneusma minus* was isolated from both healthy as well as diseased trees, albeit at a lower frequency in the healthy trees. In contrast, *Lophodermium conigenum* was isolated only from susceptible trees.

Cultivation dependent methodology has been the most commonly used in the study of endophytes (Unterseher and Schnittler 2009) although endophytic cultures commonly lack distinctive morphological characters such as reproductive structures. Morphotaxa, based on gross colony features, are considered as functional or operational taxonomic units (OTUs) (Arnold et al. 2003) supported by DNA analyses (Collado et al. 2007, Stefani and Berube 2006). Cultivation independent molecular methods permit the detection and identification of fungi and are especially relevant for endophytic fungi that are not easily culturable (Unterseher and Schnittler 2009, Zuccaro et al. 2003). DNA is extracted from plant tissues such as pine needles, and fungal DNA amplified using fungal-specific primers for various gene regions, including the rDNA Internal Transcribed Spacer (ITS) (Gherbawy and Gashgari 2013, Krabel et al. 2013). The rDNA ITS has been selected as the most informative barcode region across a wide range of fungal species (Schoch et al. 2012), even though additional gene regions will be required to separate species in particular genera (O'Donnell et al. 2010). Factors influencing this choice of barcode region included its already broad acceptance and demonstration of its utility in phylogenetic analyses and species-level discrimination for a wide range of taxa and the easy accessibility by PCR amplification using primers targeting the conserved flanking regions. These considerations have also resulted in the ITS region having the broadest representation of taxa in the international nucleotide sequence databases (INSDS), though drawbacks include a significant percentage of inadequately or incorrectly identified taxa (Tedersoo et al. 2011).

The level of sequence variation that is acceptable within a ‘species’ or OTU has been the subject of some debate. Intraspecific variation in the rDNA ITS can be as high as 5% in some species of Basidiomycota (Begerow et al. 2010). For ascomycete species, however, interspecific variation may be quite low (Begerow et al. 2010), and some species may share identical ITS sequences. Relying solely on a given level of sequence similarity to discriminate

species may result in splitting of some species, and lumping of others. (Nilsson et al. 2011) also argue against a static, predetermined level of similarity for species demarcation. Biodiversity studies based on DNA barcodes often rely on automated sequence analysis with a heavy reliance on database searching (Taylor and Houston 2011). Unless a well-curated database is available, there is a significant risk of misidentification caused by incorrectly identified sequence accessions in the INSDs (Kõljalg et al. 2013, Zhang et al. 2013). This may not be of critical importance for studies focussed mainly on biodiversity, but inferring potential roles of molecular operational taxonomic units (MOTUs) in, e.g. forest health, requires a higher degree of rigour, until a well-populated, curated fungal barcode database (Schoch et al. 2012) is available. The use of phylogenetic analyses has been proposed as a more robust method for species identification (Begerow et al. 2010) that does not depend on a previously determined cut-off level of sequence similarity.

This paper provides an overview of the fungal endophytes of *P. radiata* detected during two studies investigating the relationship between needle fungal communities and host susceptibility to Spring Needle Cast (SNC) (Prihatini et al 2014b) or to environmental variables (Prihatini et al 2014c) in Tasmania. Fungal MOTUs were detected by direct PCR amplification of fungal rDNA ITS from pine needles, cloning and Sanger sequencing. A reference culture collection of isolates from *P. radiata* needles was created to support the identification of MOTUs and phylogenetic analyses were carried out to refine initial MOTU groupings and increase confidence in identification to the level of species or other taxonomic rank, particularly for MOTUs not represented by isolates. Some of the limitations imposed by single locus barcoding are discussed.

III.2. MATERIALS AND METHODS

III.2.1. Fungal isolation and molecular confirmation of reference cultures

Green, yellow and brown needle samples were collected from trees of between three and 12 years old (y.o.). Needles were collected between December 2006 and April 2007 from 12 plantations representing the geographic and climatic ranges of Tasmanian *P. radiata* plantations (Prihatini et al. 2015b) and from a family trial examining the heritability of SNC resistance (Prihatini et al. 2015a). Needles were kept in paper bags during transportation then placed into zip lock plastic bags and stored at 4^{o}C for up to 48 h before isolations were attempted. Green, yellow, and brown needles retained on or detached from trees, and with and without any fungal apothecia were selected from the different sites.

Table 1. References isolates of *Cyclaneusma* spp., *Lophodermium* spp., *Ceuthospora pinastri* and *Strasseria geniculata* included in the DNA analyses.

Species	Culture code	Source	Origin / location	GenBank
Ceuthospora pinastri	NZFS504	Scion	New Zealand	KF013549
Cyclaneusma niveum	N215	FBRC*	Livorno, Italy	KF013553
C. niveum	N218	FBRC	Nancy, France	KF013554
C. minus 'simile'	MC103y/5	Scion	New Zealand	KF013619
C. minus 'simile'	MC300L/2c	Scion	New Zealand	KF013578
C. minus 'verum'	C348	FBRC	Pehl, Germany	KF013569
C. minus 'verum'	MC303L/6B	Scion	New Zealand	KF013598
Lophodermium sp.	NZFS796	Scion	New Zealand	KF013544
Lophodermium sp.	NZFS804	Scion	New Zealand	KF013545
L. conigenum	NZFS790	Scion	New Zealand	KF013547
L. conigenum	NZFS781	Scion	New Zealand	KF013546
Strasseria geniculata	NZFS506	Scion	New Zealand	KF013550
S. geniculata	MC7964/2	Scion	New Zealand	KF013551

*Federal Biological Research Centre for Agriculture and Forestry, Berlin.

Needles were cut into three or four sections of about 3 cm long and surface-sterilized by immersion for 1 min in 90% ethanol, rinsed in sterile water and soaked in 4% final concentration of sodium hypochlorite ($NaOCl_2$, Chem. Supply) for 5 min, then rinsed twice with sterile water. Needle fragments were dried on paper towels that had been sprayed with 90% ethanol, then placed directly onto 2% MA medium. Plates were incubated at 22 °C and inspected every two days. Mycelial growth from each needle section was subcultured onto a fresh Petri dish containing 2% MA. Subcultures were observed every three days. After about a month, cultures were grouped according to macro-morphological characters i.e. colour, shape and thickness of colony, texture, margins and growth rate (by estimating the diameter of cultures) (Stalpers 1978). Additional isolates as reference culture were obtained from Scion and FBRC (Table 1).

Mycelium was ground with a plastic pestle in 1.5 mL microcentrifuge tubes and DNA extracted using SDS buffer (Raeder and Broda 1985) and purified using glassmilk (Boyle & Lew 1995) according to Glen (2001) and used as template in PCR reactions. Fungal DNA was amplified using primers ITS1-F and ITS4 (Gardes and Bruns 1993, White, Bruns, et al. 1990). The final concentrations of reagents used in the PCR were: 67 mM Tris-HCl, pH 8.8; 16mM (NH4)2SO4 (in 10× NH4-based reaction buffer supplied by Bioline); 2.0 mM magnesium chloride (Promega); 200 μM each deoxynucleotide triphosphate (Bioline); 0.25 μM each oligonucleotide primer (Geneworks); 0.02 units μL-1 of Mangotaq DNA polymerase (Bioline); 0.2 μg μL-1 of bovine serum albumin (Fisher Biotech) to reduce enzyme inhibition that may be present in the DNA template (Kreader 1996); 10 μL of the diluted DNA in TE (1/40) as template and sterile water (Astra Zeneca) to make the volume up to 50 μL. Amplification was performed using a Peltier Thermal Cycler PTC-225 (MJ Research) or an Applied Biosystems 2720 thermocycler and the following temperature profile: 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 2 min, with a final 7

min extension at 72°C. PCR products were separated by electrophoresis on a 1% agarose gel (Fisher Biotech) at 10 volts/cm for 30 min using MI-DEAR 120 High Performance Gel System (Biokeystone). Gels were visualized on a Vilber Lourmat transilluminator after staining with ethidium bromide 0.5 μg mL-1 (MOBIO) for 20 min and images captured using a Vilber Lourmat camera (Fisher Biotech). The rDNA ITS region from at least three cultures in each morphological group, all of the singletons and additional references isolates were sequenced and analysed.

III.2.2. Direct PCR, cloning and molecular identification

In total, 48 bulk pine needle samples were collected from 12 young plantations (five y.o.) across Tasmania. Needle samples, consisting of 1, 2 and 3 year old needles and fallen needles, were selected from five trees per plantation. A more intensive sampling of a marker-aided selection trial at Oonah (eight y.o. trees) yielded 108 bulk pine needle samples, comprising of three needle age groups, from trees with four different disease severity scores in three different tree lineages, with three replicates of each needle age x disease severity x tree lineage. All needles samples were collected during spring 2007. From each bulk sample, five needles were selected from different fascicles. Approximately 1 cm needle was cut from the middle section of each needle and placed into a 1.5 mL microcentrifuge tube and stored at -80°C prior to DNA extraction.

Needles were ground using a porcelain pestle and mortar with liquid nitrogen added to facilitate grinding. DNA was extracted from *P. radiata* needles using the method outlined above for fungal isolates. DNA was amplified using primers ITS4A (Larena et al. 1999) and ITS5 (White et al. 1990). PCR conditions and thermocycler program were as specified above for amplification of fungal isolate DNA. Nested PCR was performed on samples which had insufficient product for cloning after a single round of PCR. The product of first round of

PCR using ITS4A and ITS5 was diluted 1 in 5 in TE buffer and used as a template in the second-round PCR with primers ITS1 and ITS4 (White, Bruns, et al. 1990). The thermocycler program and reagent concentrations for nested PCR were as described for the first-round PCR except the number of cycles was reduced to 20. PCR products were separated by electrophoresis on a 1% agarose gel (Fisher Biotech) at 10 volts/cm for 30 min using MI-DEAR 120 High Performance Gel System (Biokeystone).

PCR or nested PCR products from a single 50 µl reaction were pooled with replicate samples before cloning. For the samples collected from the Oonah trial, PCR products from needles of the same tree pedigree, same needle age and same disease score were pooled, resulting in 36 pooled samples from the 108 DNA extracts. From the 12 other plantations, PCR products from needles of the same age from five trees in a single plantation were pooled, resulting in 48 pooled samples from the 240 DNA extracts. Prior to cloning, PCR products were cleaned using an UltraClean™ PCR Clean-up Kit (MO BIO) according to the manufacturer's instructions. DNA was eluted in 50 µL elution buffer (MO BIO) and precipitated by addition of 2 µL of 5M NaCl and 100 µL of 100% cold ethanol, incubating on ice for 10 minutes and centrifugation at 14.000rpm for 5 min. Dried pellets were resuspended in 13 µL of TE buffer. A 3 µL aliquot of concentrated DNA was separated on a 1% agarose gel to determine the approximate concentration by comparison of band intensity with DNA size standards of known concentration. Ligation reactions were performed on the same day as the PCR and DNA clean up. Cloning reactions were performed using the pGEM®–T Easy Vector Kit (Promega) according to the manufacturer's instructions.

PCR-RFLP of cloned DNA was conducted to select the cloned bacterial colonies prior to sequencing and aimed to reduce the number of DNA samples for sequencing. A rapid DNA preparation was prepared from bacterial colonies by scraping the colony with a sterile pipette

tip or toothpick and transferring it into 200 µL of TE buffer in a 96-well PCR plate. The cells were disrupted by vortexing, cell debris pelleted by centrifugation for 1 min at 1500 rpm and 5 µL of the supernatant used as template in PCR. PCR using primers ITS1 and ITS4 was performed as outlined previously. PCR products were digested using restriction enzymes AluI (Promega) and HinFI (Promega) in a reaction with a final concentration of ingredients as follows: 60mM Tris HCl (pH7.5), 500 mM NaCl2, 60 mM $MgCl_2$ and 10 mM DTT (in 10× buffer supplied by Promega; 0.1mg mL^{-1} bovine serum albumin; 1 unit of enzyme; 5 µL of PCR product and distilled water (Astra Zeneca) up to 10 µL. Digested PCR products were separated for 2 h at 30 V cm^{-1} in a 3% high resolution agarose gel (Fisher Biotech). Gels were visualized on a Vilber Lourmat transilluminator after staining with ethidium bromide 0.5 µg mL^{-1} (MOBIO) for 20 min and images captured using a Vilber Lourmat camera (Cedex). Products of clones from the same transformation reaction were electrophoresed on the same gel to facilitate visual assessments of groups according to their RFLP patterns. Only clones with identical PCR-RFLP profiles were assigned to the same group and two or three clones from each PCR-RFLP group were randomly selected for sequencing.

III.2.3. DNA Sequencing and Sequence analysis

PCR products of fungal isolates, including reference isolates (Table 1), and PCR products of selected clone samples were sequenced. DNA sequencing of PCR products was outsourced to Macrogen Inc (http://dna.macrogen.com/eng/). Chromatograms were viewed in ChromasPro version 1.34 software and edited to remove poor quality sequences at each end. A BLAST (Basic Local Alignment Search Tool) search of the International Nucleotide Sequence Databases (non-redundant database accessed from the National Centre for Biotechnology Information website - http://www.ncbi.nlm.nih.gov/) retrieved sequences of high similarity (Altschul, Gish, et al. 1990). Sequences were grouped according to BLAST search results,

and aligned with sequences of high similarity using ClustalW (Thompson, Higgins, et al. 1994) and default parameters (gap opening penalty 15, gap extension penalty 6.66, IUB weight matrix and transition weight of 0,5). If single nucleotide polymorphisms occurred between colonies or clones, chromatograms were rechecked to confirm these.

III.2.4. Phylogenetic analysis

Isolates or clones with less than 2% sequence variation (equivalent to p-distance in MEGA) were grouped into MOTUs. The fungal isolates or MOTUs were identified to the lowest possible taxonomic level based on sequence similarity to known fungi from the INSDS or reference isolates. Each set of aligned sequences was scrutinised to identify variants that differed by more than one or two bp within MOTUs. A representative sequence was selected for each MOTU, or of each variant within a MOTU, where these existed, based on length of high quality sequence, and used for MOTU identification. BLAST results for each MOTU were scrutinised to ascertain which were most robustly identified, based on criteria published in (Glen, Yuskianti, et al. 2014) and the selected accessions used as reference sequences for that MOTU. Where phylogenetic analyses were carried out, one or two sequences from more distantly related taxa were also included as outgroups. All sequences for each phylogenetic analysis were aligned using CLUSTALW program in BioEdit software version 7.0.9.0 (Hall 1999), with default parameters as provided above. The aligned sequences were used for Maximum Likelihood phylogenetic analysis using DNAml version 3.6a2.1 from the PHYLIP package (Felsenstein 1989) with default parameters. Bayesian trees were also constructed using MrBayes version 3.2 (Huelsenbeck and Ronquist 2001), and running for 500,000 to 1,600,000 generations to obtain average standard deviation below 0.01. Phylogenetic trees were viewed and rooted using out-group taxa in TreeView software (Page 1996), and labels edited in Powerpoint.

III.3. RESULTS

III.3.1. Characterization of fungal cultures

A total of 544 isolates were obtained from 180 needle samples. The cultures were grouped according to macro-morphological characters, resulting in 26 morphological groups that each consisted of at least three cultures (Supplementary Table 1) and 14 singletons. After phylogenetic analysis, the appropriate identity of each OTU was confirmed which resulted in 35 OTUs, including three e OTUs in the Basidiomycota.

The identities of the OTUs were compared to the morphological grouping of cultures. In three cases, different morphological groups were given the same OTU identification (Supplementary Table 1); two morphologically different groups (groups 3 and 4) were determined to be *Lophodermium pinastri*, another two morphological groups (groups 5 and 6) were identified as *Lophodermium* aff. *conigenum* and a third pair were identified as *Phoma* sp. (groups 12 and 13).

Some cultures were identified to species or genus level but some were only identified to family or order (Pezizales, Helotiales and Xylariaceae) or phylum (Basidiomycota species). The inability to identify several cultures to species or genus level was due to:

High sequence similarity (98%-100%) to several different species within a broader taxonomic group (e.g. Helotiales sp. 1).

High sequence similarity (> 90%) to other fungi that were identified only to order but less than 90% to known fungal species (Helotiales sp. 2 and Xylariales sp. 1).

In contrast to morphological variation within an OTU, some morphological groups were separated into more than one distinct clade in phylogenetic analysis; those different

clades were considered to represent distinct species. Examples include a morphological group that is comprised of two distinct clades within *Coniochaeta*, another comprised of four distinct clades within Xylariaceae and a third comprised of 2 distinct clades within Xylariales.

III.3.2. Identification of needle fungal MOTUs

The starting point of 98% sequence similarity for grouping of MOTUs produced 130 MOTUs from 1303 sequences derived from fungal isolates and DNA extracted from *P. radiata* needles. Phylogenetic analysis helped to refine the discrimination and identification of MOTUs. After further analyses, including database searching, sequence alignment and phylogenetic analyses, a total of 167 MOTUs were recognised from isolates and DNA fragments amplified from pine needles (Table 2). Of these, 47 MOTUs were represented by fungal isolates. Forty six of the cloned sequences were derived from Basidiomycota while 1257 were from Ascomycota. The Ascomycota were classed into 15 orders, one group of unknown Saccharomycetes, a *Phaeomoniella* sp. (Ascomycota, incerta sedis) and another seven OTUs of uncertain placement in the Ascomycota. Basidiomycota were classed into seven orders, leaving seven MOTUs with uncertain placement in the Basidiomycota.

Phylogenetic analyses were aimed at different taxonomic levels depending on the availability of ITS sequences from closely related species. For those MOTUs for which closely related taxa could not be identified, analyses were conducted at higher taxonomical levels, such as Family (Mycosphaerellaceae and Teratosphaeriaceae). These analyses were not intended to be robust phylogenetic analyses of the entire family, order or class, but included species with the highest sequence similarity to the MOTU being examined, additional representatives of the family, order or class, and one or more outgroups. For MOTUs with a high level of sequence similarity to a known fungal species and for potentially

pathogenic species, including *Lophodermium conigenum* (Figure 5) and *L. pinastri* (Figure 6) a selection of closely related species within the same genus were included in the analysis.

III.3.3. Fungal diversity in *P. radiata* needles

Only 10 of the 153 MOTUs were detected by direct DNA amplification and fungal isolation, 25 by isolation only, and the remaining 118 were detected by DNA only. The vast majority of MOTUs were uncommon, with only 13 detected in 8 or more of the 70 bulk samples, each of which included needles from three to five trees. Six of the commonly detected MOTUs consisted of species associated with pine needles in previous studies; *Cyclaneusma minus* 'verum', *C. minus* 'simile', *Dothistroma septosporum, Lophodermium pinastri, Strasseria geniculata* and *Sydowia polyspora.* All but *D. septosporum* were also isolated in this study. The remaining seven commonly detected MOTUs included five unidentified Teratosphaeriaceae and two unidentified Ascomycota.

The number of MOTUs in each Class or Division and their prevalence were calculated and are presented in Table 3. The most frequently detected group in this study was Dothideomycetes, while Sordariomycetes was the third most frequently detected group, but the most highly represented by isolation. Leotiomycetes was the second most frequently detected group, by culturing as well as by DNA amplification.

Table 2. Fungi detected in *P. radiata* needles by isolation (CL = cultures) and PCR amplification followed by cloning (NY = needles of 5 y.o. trees and NO = needles from 8 y.o. trees. NO and NY indicate the number of needle samples (8 y.o. and 5 y.o., respectively) in which the OTU was detected and CL the number of culturesobtained inthis study.

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
Dothideomycetes						
Botryosphaeriales; Family undet.	Botryosphaeriales sp. 1	0	2	0	KM216369	Sequence similarity of 98% to AY843079, an unnamed fungal isolate from rock surfaces, and up to 86% to several species of Botryosphaeriales, including KC869997, *Diplodia seriata* culture-collection CBS:134701 and FJ888478, *Diplodia alatafructa* CMW22721.
	Botryosphaeriales sp. 2	2	0	0	KM216352	Sequence similarity of 93% to AY843079 and up to 88% to several species of Botryosphaeriales, including DQ458889, *Botryosphaeria obtusa* CBS119049 and EF127892, *Diplodia seriata* strain CBS 200.49.
	Botryosphaeriales sp. 3	1	0	0	KM216350	Sequence similarity up to 90% to several species of Botryosphaeriales, including EU603293, *Botryosphaeria viticola* and EF591921, *Dothiorella moneti.*
Capnodiales;						
Family undet.	Capnodiales sp. 1	0	1	0	KJ406762	Phylogenetic analysis, Figure 1
	Capnodiales sp. 2	1	1	0	KJ406757	Phylogenetic analysis, Figure 1
	Capnodiales sp. 3	1	0	0	KM216329	Phylogenetic analysis, Figure 1

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
	Phaeotheca fissurella	3	13	0	KM216349	Sequence similarity of 99% to AJ244255.1, *Phaeotheca fissurella* CBS 520.89, less than 90% similarity to any other sequence from identified fungi.
Davidiellaceae	*Davidiella* sp.	8	3	0	KM216336	Sequence similarity of 99.8% to a range of *Davidiella* and *Cladosporium* (anamorph of *Davidiella*) species, including, *D. tassiana*; *D. macrospora*; *C. ramotenellum; C. cucumerinum.*
	Davidiellaceae sp. 1	3	0	0	KM216333	Sequence similarity of 93-94% to four species of *Rachicladosporium.*
	Davidiellaceae sp. 2	1	0	0	KM216347	Sequence similarity of 93-95% to four species of *Rachicladosporium*.
Mycosphaerellaceae	*Dothistroma septosporum*	2	16	0	KJ406805	Phylogenetic analysis, Figure 2
	Mycosphaerellaceae sp. 1	3	2	0	KJ406789	Phylogenetic analysis, Figure 2
	Mycosphaerellaceae sp. 2	0	1	0	KJ406794	Phylogenetic analysis, Figure 2
	Mycosphaerellaceae sp. 3	0	1	0	KJ406799	Phylogenetic analysis, Figure 2
	Phaeothecoidea sp. 1	4	0	0	KJ406802	Phylogenetic analysis, Figure 2
	Phaeothecoidea sp. 2	1	0	0	KJ406792	Phylogenetic analysis, Figure 2
	Phaeothecoidea sp. 3	0	2	0	KJ406797	Phylogenetic analysis, Figure 2
	Pseudocercospora sp. 1	1	0	0	KJ406795	Phylogenetic analysis, Figure 2
	Ramularia stellenboschensis	1	0	0	KJ406791	Phylogenetic analysis, Figure 2

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
Teratosphaeriaceae	*Devriesia* sp.	0	1	0	KM216374	Sequence similarity of 93% to AY692088.1, *Devriesia thermodurans.*
	Teratosphaeria aff. associata	2	0	0	KJ406767	Phylogenetic analysis, Figure 1
	Teratosphaeria aff. capensis	1	4	0	KJ406771	Phylogenetic analysis, Figure 1
	Teratosphaeria aff. parva	1	4	0	KJ406775	Phylogenetic analysis, Figure 1
	Teratosphaeria aff. suttonii	1	0	0	KJ406763	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 01	0	4	0	KJ406774	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 02	0	2	0	KJ406778	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 03	3	12	0	KM216368	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 04	1	7	0	KJ406765	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 05	2	0	0	KJ406777	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 06	2	0	0	KJ406766	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 07	1	0	0	KJ406768	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 08	2	2	0	KJ406760	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 09	1	0	0	KJ406759	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 10	3	0	0	KJ406770	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 11	0	1	0	KJ406764	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 12	0	1	0	KJ406783	Phylogenetic analysis, Figure 1

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
	Teratosphaeriaceae sp. 13	0	6	0	KJ406782	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 14	4	0	0	KJ406779	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 15	0	4	0	KJ406758	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 16	1	5	0	KJ406776	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 17	1	0	0	KJ406785	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 18	2	4	0	KJ406786	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 19	1	0	0	KJ406787	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 20	0	1	0	KM216331	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 21	2	6	0	KJ406780	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 22	0	3	0	KJ406769	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 23	9	33	0	KJ406781	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 24	1	5	0	KJ406784	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 25	0	1	0	KM216373	Phylogenetic analysis, Figure 1
	Teratosphaeriaceae sp. 26	15	2	0	KJ406761	Phylogenetic analysis, Figure 1
Dothideales; Dothioraceae	*Aureobasidium pullulans*	2	2	0	KJ407004	Phylogenetic analysis, Figure 3

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
	Sydowia polyspora	10	2	2	KJ407006	Phylogenetic analysis, Figure 3
Family undet.	Dothideales sp. 1	1	0	0	KJ406832	Phylogenetic analysis, Figure 3
Pleosporales						
Didymellaceae	*Leptosphaerulina* sp. 1	1	0	0	KJ406837	Sequence similarity of up to 99.8% to accessions from several *Leptosphaerulina* spp., including KJ796400, *L. chartarum.*
	Dothideomycetes sp. 2	1	1	0	KJ406840	Sequence similarity of 98-99% to a range of Dothideomycete spp., including AJ279448, *Epicoccum nigrum.*
	Didymellaceae sp. 2	0	3	0	KM216364	Sequence similarity of 98% to a range of *Peyronellaea* and *Phoma* species, including GU237818, *Peyronellaea aurea.*
Lophiostomataceae	*Lophiostoma corticola*	0	2	0	KJ407013	Sequence similarity of 99.5% to multiple accessions of *Lophiostoma corticola*, including HE774481.
Montagnulaceae	*Paraphaeosphaeria michotii*	3	0	0	KJ406849	Sequence similarity of 99.8% to multipole accessions of *Paraphaeosphaeria michotii*, including JX496079, and 98% similarity to other *Paraphaeosphaeria* species.
Phaeosphaeriaceae	*Phaeosphaeria* sp. 2	0	1	0	KM216376	Sequence similarity of 98-99% to a range of *Phaeosphaeria* species, including JX981464, *P. culmorum.*
Pleosporaceae	*Allewia eureka*	0	1	0	KM216382	Sequence similarity of 99% to JN383490, *Embellisia eureka* (syn. *Allewia eureka*), and up to 97% sequence similarity to several other species of *Alternaria* including, KC584222, *A.*

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
						triglochinicola CBS 119676.
Sporormiaceae	*Sporormiella intermedia*	1	0	0	KJ406863	Phylogenetic analysis, Figure 4
	Preussia/Sporormiella sp. 1	0	1	0	KJ406868	Phylogenetic analysis, Figure 4
	Preussia/Sporormiella sp. 2	0	0	2	KJ407017	Phylogenetic analysis, Figure 4
	*Preussia/Sporormiella*sp. 3	1	0	0	KJ406867	Phylogenetic analysis, Figure 4
	Preussia/Sporormiella sp. 4	0	1	0	KJ406866	Phylogenetic analysis, Figure 4
	*Preussia/Sporormiella*sp. 5	0	1	0	KJ407014	Phylogenetic analysis, Figure 4
	Preussia/Sporormiella sp. 6	0	1	0	KJ406865	Phylogenetic analysis, Figure 4
Family undet.	Pleosporales sp. 1	0	5	0	KJ406858	Sequence similarity of up to 97% to several species of *Peyronellaea* and *Phoma*, including GU237847 *Peyronellaea obtusa.*
	Pleosporales sp. 2	2	1	7	KJ407009	Sequence similarity of 99-100% to species of *Microsphaeropsis*, *Phoma,* and *Atradidymella,* including JX681101, *Microsphaeropsis olivacea.*
	Pleosporales sp. 3	0	1	0	KJ406852	Sequence similarity of up to 94% to a range of Pleosporales species, including EU295638, *Paraconiothyrium brasiliense.*
	Pleosporales sp. 4	0	2	0	KJ406853	Sequence identity with a range of Pleosporales species, including EU295638, *Paraconiothyrium brasiliense.*
	Pleosporales sp. 5	0	6	0	KJ406856	Sequence similarity of up to 93% to a range of Pleosporales species, including AB554112, *Prosthemium stellare.*

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
	Pleosporales sp. 6	0	1	0	KM216359	Sequence similarity of up to 93% to a range of Pleosporales species, including EU295638, *Paraconiothyrium brasiliense.*
Eurotiomycetes						
Chaetothyriales;						
Herpotrichiellaceae	*Exophiala eucalyptorum*	2	0	0	KJ406880	Sequence similarity of 99.6-100% to two accessions of *Exophiala eucalyptorum*, including EU035417, sequence similarity of up to 96% to other Chaetothyriales species.
	Exophiala sp. 1	0	1	0	KJ406883	Sequence similarity of 98% to DQ914677, *Exophiala* sp., sequence similarity of up to 83% to other Chaetothyriales species.
	Exophiala sp. 2	0	1	0	KJ406881	Sequence similarity of 95-96% to two accessions of *Exophiala eucalyptorum*, including EU035417, sequence similarity of up to 90% to other Chaetothyriales species.
Family undet.	Chaetothyriales sp. 1	3	0	0	KJ406877	Sequence similarity of 80-89% to a range of *Phaeococcomyces* species, including KJ152783, *P. mexicana.*
	Chaetothyriales sp. 2	4	1	0	KJ406875	Sequence similarity up to 93% to a range of Chaetothyriales spp., including EU035415, *Cyphellophora hylomeconis.*
	Chaetothyriales sp. 3	1	0	0	KJ406884	Sequence similarity of 80-87% to a range of *Phaeococcomyces* species, including KJ152783, *P. mexicana.*
Eurotiales;						

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
Trichocomaceae	*Aspergillus*sp. 1	0	1	0	KJ406885	Sequence similarity of 93-94% to several accessions of *A. conicus*, including EF652039, up to 91% similarity to other *Aspergillus* species.
	Aspergillus sp. 2	0	6	0	KJ406898	Sequence similarity of 99% to several species of *Aspergillus*, including AY373877, *A. ustus*.
	Aspergillus sp. 3	0	0	1	KJ407020	Sequence similarity of 99% to several species of *Aspergillus*, including KJ775589, *A. versicolor*.
	Penicillium sp.1	1	0	0	KJ406888	Sequence similarity of 96% to several species of *Penicillium*, including KJ775589, *P. chrysogenum*.
	Penicillium sp. 2	4	0	0	KJ406887	Sequence similarity of 96% to several species of *Penicillium*, including AY373906, *P. corylophilum*.
	Penicillium sp. 3	1	0	0	KJ406890	Sequence similarity of 96% to several species of *Penicillium*, including AF033463, *P. namyslowskii*.
	Penicillium sp. 4	1	0	0	KJ406894	Sequence identity with several species of *Penicillium*, including KJ775635, *P. glabrum.*
Family undet.	*Penicillium* sp. 5	0	0	3	KJ407018	Sequence similarity of 95% to several species of *Penicillium*, including AY373898, *P. brevicompactum*.
Lecanoromycetes						
Acarosporales;						
Family undet.	Acarosporales sp. 1	0	1	0	KM624595	Sequence similarity of up to 88% to several *Acarospora* species, including GU184115.1, *A. rosulata.*

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
Lecanorales;						
Family undet.	Lecanorales sp	1	0	0	KM624596	Up to 82% sequence similarity to a range of Lecanorales sp., including AY541255.1, *Lecanora leptyrodes.*
Leotiomycetes						
Helotiales;						
Dermateaceae	*Mollisia* sp. 1	0	0	2	KM216327	Over 99% similarity to several species of *Mollisia*, including *M. melaleuca* (AY259136), *M. cinerea* (AY259135), and *M. fusca* (AY259137).
Helotiaceae	*Varicosporium* aff. *elodeae*	1	0	0	KM216334	Sequence similarity of 98% to several accessions of *Varicosporium elodeae,* including JN655610.1.
Sclerotiniaceae	*Torrendiella eucalypti*	2	0	0	KM216335	Sequence similarity of 99% to AY755335, *Torrendiella eucalypti*, and 95% sequence similarity to several other species of *Torrendiella.*
Family undet.	*Cyclaneusma minus* 'simile'	9	17	2	KJ406913	Sequence identity with reference isolates of *Cyclaneusma minus* 'simile' and 90-91% sequence similarity to *C. minus* 'verum' and *C. niveum.*
	Cyclaneusma minus 'verum'	8	2	3	KJ407021	Sequence identity with reference isolates of *Cyclaneusma minus* 'verum', 99% sequence similarity to *C. niveum* and 90% sequence similarity to *C. minus* 'simile'.
	Helotiales sp. 1	0	0	4	KM216322	Sequence similarity of up to 98% to several Helotiales species,

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
						including *Cadophora malorum* and *Mollisia dextrinospora.*
	Helotiales sp. 2	0	0	2	KM216321	Sequence similarity of up to 93% to several Helotiales species, including *Hymenoscyphus monotropae* and *Cyathicula microspora.*
	Helotiales sp. 3	0	1	0	KM216383	Sequence similarity of up to 86% to several Helotiales species, including *Arachnopeziza aurata.*
	Helotiales sp. 4	1	0	0	KM216351	Sequence similarity of up to 93% to several Helotiales species, including *Xenopolyscytalum pinea* and *Cadophora* sp.
	Helotiales sp. 5	0	1	0	KM216379	Sequence similarity of 99% to HQ608100.1, unidentified ascomycete, and up to 94% to several Helotiales species including EU940165.1, *Cyathicula microspora.*
	Meliniomyces sp.	1	0	0	KM216335	Sequence similarity of 99.8% to several accessions of *Meliniomyces bicolor,* including HQ157926.1.
Phacidiaceae	*Ceuthospora pinastri*	2	1	1	KJ406933	Sequence identity with reference isolate NZFS504, 99% sequence similarity to *Strasseria geniculata,* KF013550.
Rhytismatales;						
Rhytismataceae	*Lophodermium aff. conigenum*	4	0	21	KJ407029	Phylogenetic analysis, Figure 5; Supplementary material
	Lophodermium pinastri	13	12	78	KJ407045	Phylogenetic analysis, Figure 6; Supplementary material
Family undet.	*Fulvoflamma* sp.	1	0	0	KM216341	Sequence similarity of 92% to DQ195779, *Fulvoflamma eucalypti* CPC 11243, and up to 99% to unknown Leotiomycetes species.
Order undet.,	Leotiomycetes sp. 1	3	0	0	KJ406966	Sequence similarity of 90-91% to a range of *Collophora* spp.,

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
Family undet.						including JN808839, *C. hispanica.*
	Leotiomycetes sp. 2	0	1	0	KJ406976	Sequence similarity of up to 93% to a range of Leotiomycete spp., including HQ533008, *Claussenomyces* sp.
Pezizomycetes						
Pezizales;						
Family undet.	Pezizales sp. 1	0	0	2	KJ407055	Sequence similarity of 95% to EU552114, *Conoplea fusca* CBS 113475 and up to 93% to several species of *Plectaria.*
	Pezizales sp. 3	0	0	1	KJ407058	Sequence similarity of 80% to unknown Pezizomycetes species and *Pseudoplectania nigrella.*
Pezizaceae	*Chromelosporium carneum*	0	0	2	KJ407060	Sequence identity with several accessions of *Chromelosporium carneum*, including JF440586 and up to 91% similarity to other Pezizaceae spp.
Saccharomycetes						
Saccharomycetales;						
Family undet.	*Candida sake*	0	1	0	KM216377	Sequence similarity of 99% to AJ549822, *Candida sake*, and up to 90% similarity to several other *Candida* species.
Sordariomycetes						
Hypocreales;						
Nectriaceae	*Fusarium* sp.	0	0	1	KM216320	Sequence similarity of up to 98% to FJ228190 *Fusarium lateritium* and up to 96% to several other *Fusarium* and

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
						Gibberella species.
Coniochaetales;						
Coniochaetaceae	*Coniochaeta* sp. 1	0	0	73	KJ407067	Phylogenetic analysis, Figure 7.
	Coniochaeta sp. 2	0	0	4	KJ407066	Phylogenetic analysis, Figure 7.
Sordariales;						
Lasiosphaeriaceae	*Bagadiella* sp.	1	0	0	KM216344	Sequence similarity of 98-99% to several *Bagadiella* species, including JF951141, *B. victoriae* CPC 17688; GQ303269, *B. lunata* CBS 124762 and JF951142, *B. koalae* CPC 17682.
	Fimetariella rabenhorstii	0	0	9	KJ407070	Sequence similarity of 99-100% to multiple accessions of *Fimetariella rabenhorstii*, including KM519659 and up to 93% to other Sordariomycete spp.
	Lasiosphaeria sp.	0	0	2	KJ407064	Sequence similarity of 97-99% to several accessions of *Lasiosphaeria lanuginosa* and *L. ovina*, including AY587916 and up to 93% to other *Lasiosphaeria* spp.
Family undet.	Sordariales sp.	1	0	0	KJ406980	Sequence similarity of up to 90% to a range of Sordariales spp., including GQ154539, *Coniochaeta africana.*
Xylariales;						
Amphisphaeriaceae	*Pestalotiopsis* sp. 1	0	0	1	KJ406990	Sequence similarity of up to 99.4% to a range of *Pestalotiopsis* spp., including EU342212, *P. neglecta*.
	Pestalotiopsis sp. 2	1	0	0	KJ406983	Sequence similarity of up to 98% to a range of *Pestalotiopsis* spp., including EU552147, *P. maculiformans.*

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
Xylariaceae	*Anthostomella pinea*	0	1	3	KJ406992	Sequence similarity of 99.4% to HQ599578, *Anthostomella pinea*, less than 90 sequence similarity to sequences from any other identified fungi available on the INSDs.
	Biscogniauxia sp	0	0	3	KJ406987	Sequence similarity of 99% to JN225897, *Biscogniauxia* sp., and up to 96% to several *Biscogniauxia* species, including JX507805, *B. uniapiculata*.
	Nemania diffusa	0	0	13	KJ406996	Sequence similarity of 98-99% to multiple accessions of *Nemania diffusa*, including DQ68238.
	Rosellinia sp.	0	0	1	KJ406999	Sequence similarity of up to 94% to several *Rosellinia* species, including KC477236, *R. corticium.*
	Xylaria castorea	0	0	3	KJ406994	Sequence identity with three accessions of *Xylaria castorea*, including JN225908, and up to 97 sequence similarity with a range of other *Xylaria* species.
	Xylariaceae sp. 1	0	0	19	KJ407000	Sequence similarity of 99% to JN225905.1, an unidentified Xylariaceae, and 86-91% to several species of *Hypoxylon*, including JQ009321, *H. vinosopulvinatum.*
	Xylariaceae sp. 2	0	0	7	KJ406985	Sequence similarity of up to 85% to several *Annulohypoxylon* and *Hypoxylon* species, including EF026139, *Annulohypoxylon squamulosus.*
	Xylariaceae sp. 3	0	0	1	KJ406986	Sequence similarity of 99% to JN225894, an unidentified *Xylariaceae*, and up to 93% to several *Hypoxylon* species, including JN979427, *H. hypomiltum.*
	Xylariaceae sp. 4	0	0	1	KJ406984	Sequence similarity of up to 83% to several *Hypoxylon* species, including KC968932, *H. rickii.*

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
Ascomycetes;						
Order and family undet.	Ascomycete sp. 1	3	7	0	KM216353	High sequence similarity in the 5.8S region only to a range of ascomycete species including AY843154.1, *Phaeococcomyces nigricans.*
	Ascomycete sp. 2	0	11	0	KM216358	Sequence similarity of 95% to AJ244255.1, *Phaeotheca fissurella* CBS 520.89, up to 88% similarity to a range of other genera including *Teratosphaeria.*
	Ascomycete sp. 3	1	0	0	KM216354	High sequence similarity in the 5.8S region only to a range of ascomycete species including AY843154.1, *Phaeococcomyces nigricans,* up to 87% similarity to several uncultured ascomycete clones, including FR682186.1.
	Ascomycete sp. 4	0	1	0	KM216378	High sequence similarity in the 5.8S region only to a range of ascomycete species including FJ172275.1, *Aquapoterium pinicola.*
	Ascomycete sp. 5	0	1	0	KM216363	High sequence similarity in the 5.8S region only to a range of ascomycete species including HQ115663.1, *Pseudotaeniolina globosa.*
	Ascomycete sp. 6	0	1	0	KM216360	High sequence similarity in the 5.8S region only to a range of ascomycete species including JN628167.1, *Scleroconidioma sphagnicola.*
	Ascomycete sp. 7	1	0	0	KJ406845	High sequence similarity (up to 96%) in the 5.8S region only with a range of Ascomycete species, including KF891885,

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
						Aureobasidium pullulans.
	Strasseria geniculata	2	6	3	KJ407023	Sequence identity with reference isolates NZFS506 and MC7964/2, 99% similarity to *Ceuthospora pinastri* KF013549.
Agaricomycetes						
Agaricales;						
Physalacriaceae	*Armillaria* aff. *luteobubalina*	2	0	0	KM624597	Sequence similarity of 98% to several accessions of *Armillaria luteobubalina,* including AF394916.1,
Strophariaceae	*Pholiota multicingulata*	2	0	0	KM216337	Sequence identical to HQ533029, *Pholiota multicingulata,* and up to 93% similarity to several other *Pholiota* species.
Boletales;						
Serpulaceae	*Serpula himantioides*	2	0	0	KM216346	Sequence similarity of 99.5% to many accessions of *Serpula himantioides,* including GU187545.1 and 96% to *S. lacrymans.*
Cantharellales;						
Ceratobasidiaceae	*Ceratobasidium* sp	0	0	2	KM216319	Sequence similarity of 95% to AJ427401 *Ceratobasidium papillatum,* and up to 92% similarity to several other *Ceratobasidium* and *Rhizoctonia* species.
Tremellomycetes						
Cystofilobasidiales;						

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
Cystofilobasidiaceae	*Udeniomyces pyricola*	0	1	0	KM216371	Sequence similarity of 99% to AF44402.1, *Udeniomyces pyricola*, 98% to AY841862, *U. pseudopyricola*, and up to 85% similarity to a range of other *Udeniomyces* species.
Tremellales;						
Tremellaceae	*Cryptococcus* aff. *amylolyticus*	1	0	0	KM216348	Sequence similarity of 97% to several accessions of *Cryptococcus* aff. *amylolyticus*, including EF363150.1.
	Cryptococcus sp. 1	1	0	0	KM216340	Sequence similarity of up to 99.6% to several accessions of *Cryptococcus wieringaes*, including FN824493.1 and up to 96% to several other *Cryptococcus* species.
	Cryptococcus sp. 2	1	0	0	KM216339	Sequence similarity of up to 87% to several species of *Cryptococcus*, including JN942258.1, *C. aerius.*
	Cryptococcus victoriae	2	1	0	KM216343	Sequence similarity of up to 99.8% to many accessions of *Cryptococcus victoriae*, including HQ615694.1
	Tremella sp.	2	2	0	KM216338	Sequence similarity of 99% to DQ242633.1, *Tremella* sp., and up to 83% similarity to a range of identified *Tremella* species.
	Tremellaceae sp. 1	1	0	0	KM624601	Sequence similarity of up to 87% to a range of Tremellaceae, including *Cryptococcus fagi* and *Tremella neofoliacea.*
	Tremellaceae sp. 2*	1	0	0	KM624602	Up to 90% sequence similarity to a range of Tremellaceae species, including AF444404, *Fellomyces horovitziae*.
Trichosporonaceae	*Trichosporon dulcitum*	0	1	0	KM216365	Sequence similarity of 99% to Af444428.1, *Trichosporon dulcitum*, and 98% to several other *Trichosporon* species.

Class; **Order; Family**	**OTU**	**NY**	**NO**	**CL**	**GenBank Accession**	**Criterion for identification**
Basidiomycetes; Order and family undet.	Basidiomycete sp. 1	1	0	0	KM624603	Up to 92% sequence similarity to several species of *Rhodotorula* and *Sporobolomyces,* including FN424107.1, *R.* aff. *diffluens.*
	Basidiomycete sp. 2	2	0	0	KM624606	High sequence similarity in the 5.8S region only to a range of basidiomycete species including JN620354, *Bensingtonia* sp.
	Basidiomycete sp. 5	0	0	1	KM624590	Up to 92% sequence similarity to several uncultured fungal clones, and 86% to a range of basidiomycete species, including JF908470.1, *Mycena senesii.*
	Basidiomycete sp. 7	2	0	1	KM624589	Sequence similarity of up to 86% to a range of basidiomycete species, including *Armillaria novae-zelandiae.*

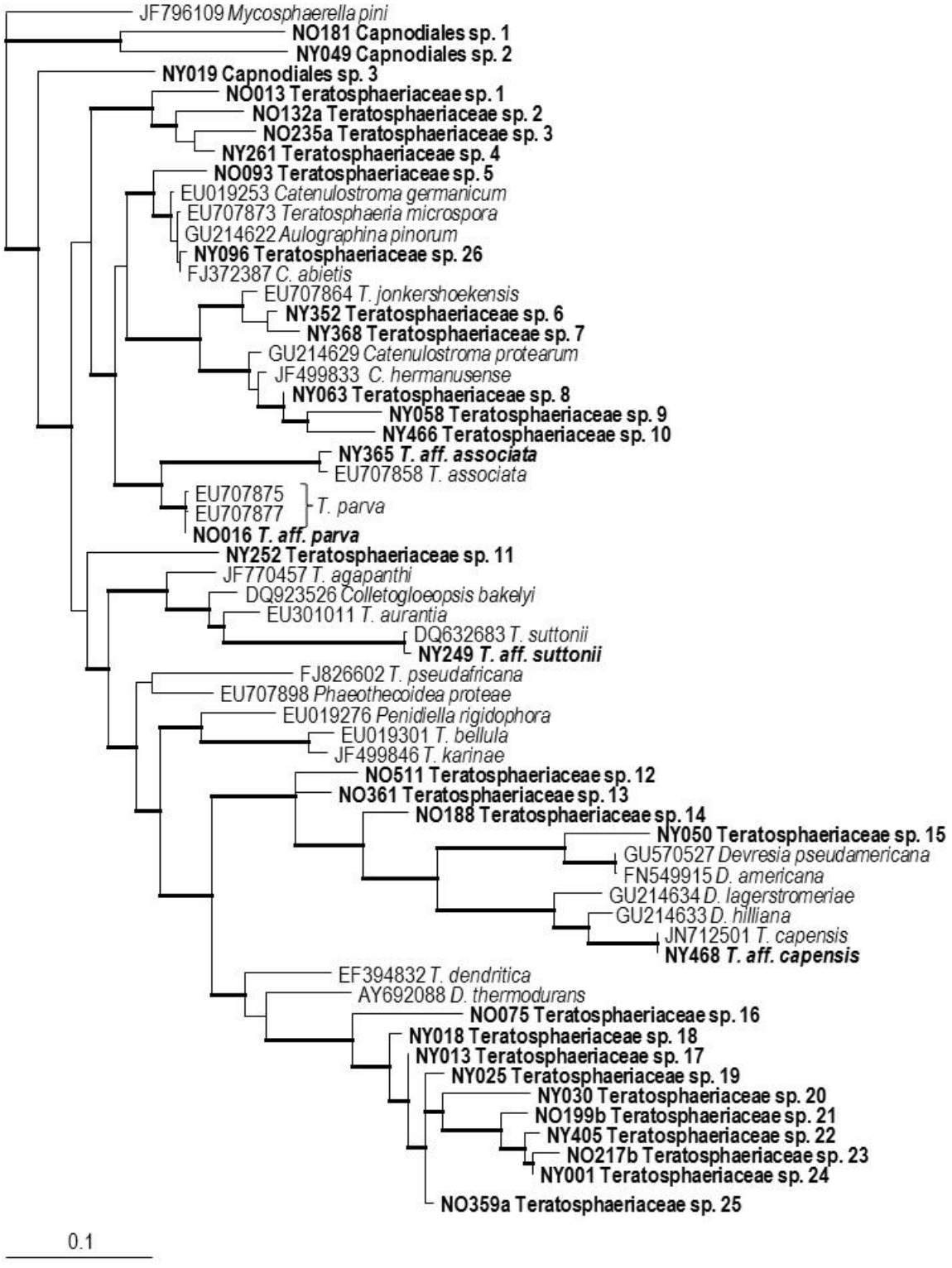


Figure 1. Maximum Likelihood analysis of ITS sequences of Teratosphaeriaceae species detected from needles of five year old (NY) and eight year old (NO) trees, including the highest scoring sequences from BLAST searches of the INSDs, rooted to *Mycosphaerella pini*. LnL = -7827.68712, bar represents 10% expected variation. Branches in bold represent significance at P<0.01.

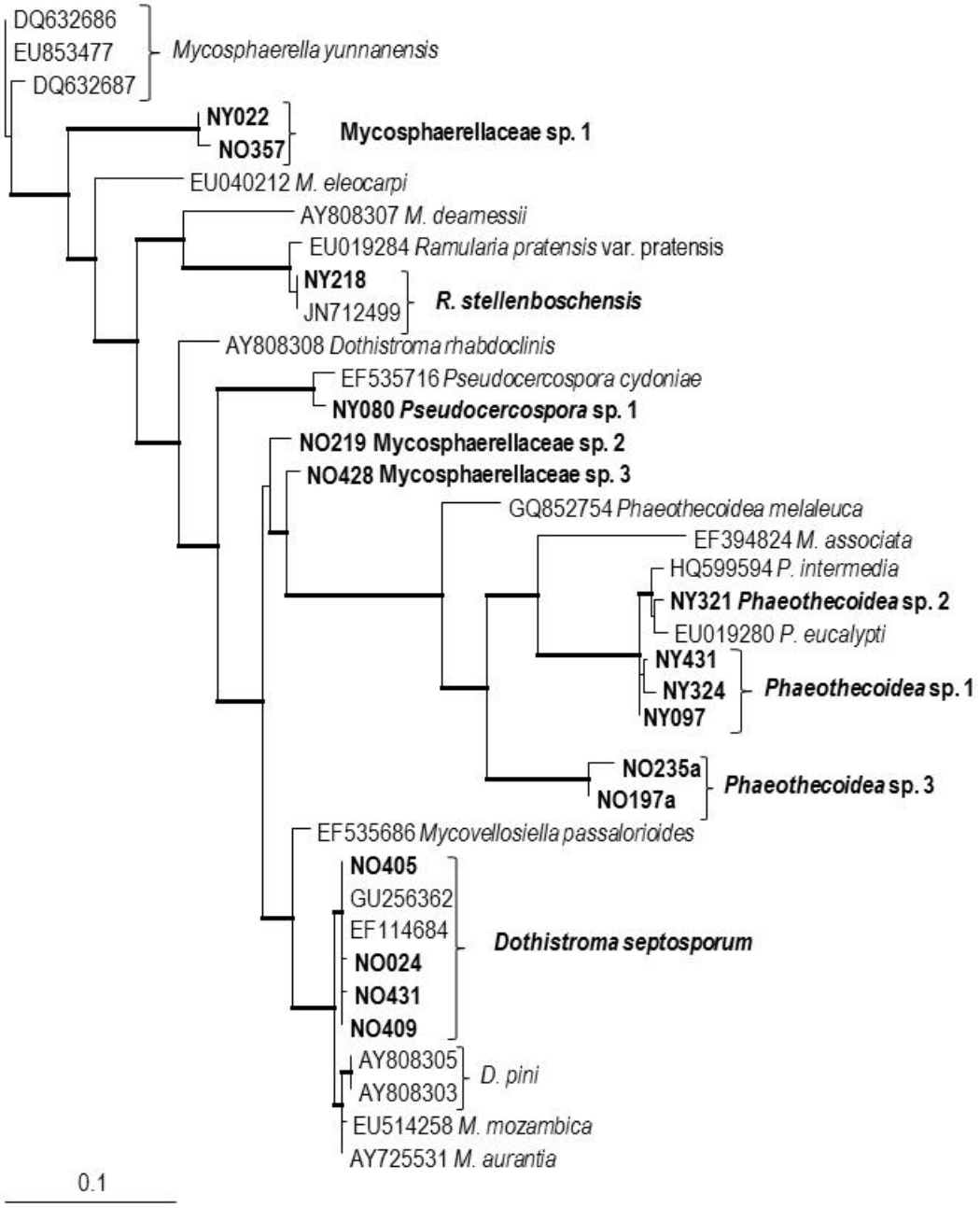


Figure 2. Maximum likelihood analysis of ITS sequences of Mycosphaerellaceae species amplified from needles of five year old (NY) and eight year old (NO) trees, including the highest scoring sequences from BLAST searches of the INSDs, rooted to *Mycosphaerella yunnanensis*. LnL = -2950.39512, bar represents 10% expected variation. Branches in bold represent significance at P<0.01.

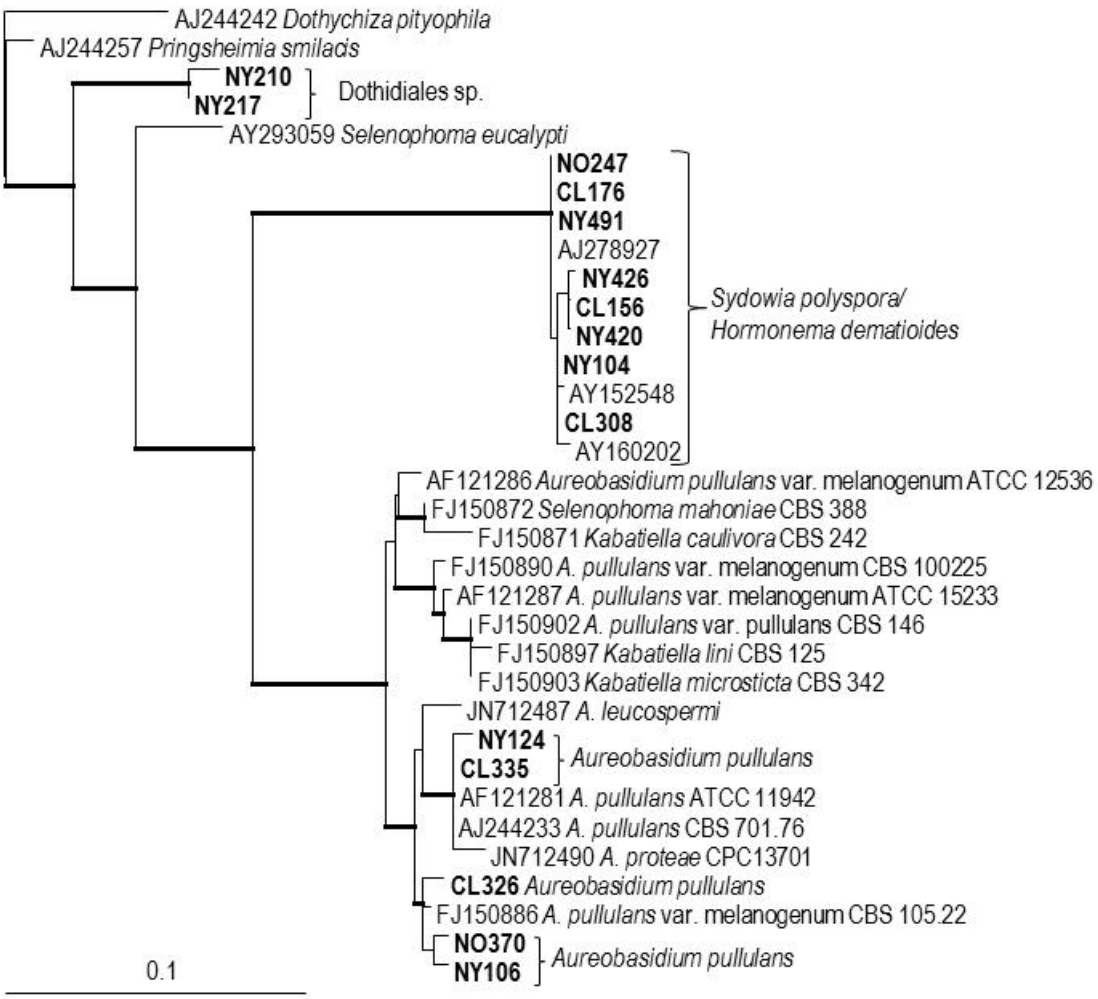


Figure 3. Maximum Likelihood analysis of ITS sequences of Dothidiales species (*Sydowia polyspora* and *Aureobasidium pullulans*) amplified from cultures (CL) and needles of five year old (NY) and eight year old (NO) trees, including the highest scoring sequences from BLAST searches of the INSDs, rooted to *Dothichiza pityophila*. LnL = -2100.79916, bar represents 10% expected variation. Branches in bold represent significance at P<0.01. Note that *Hormonema dematioides* is an anamorph of *Sydowia polyspora* and that *Kabatiella lini* is a synonym of *Aureobasidium pullulans.*

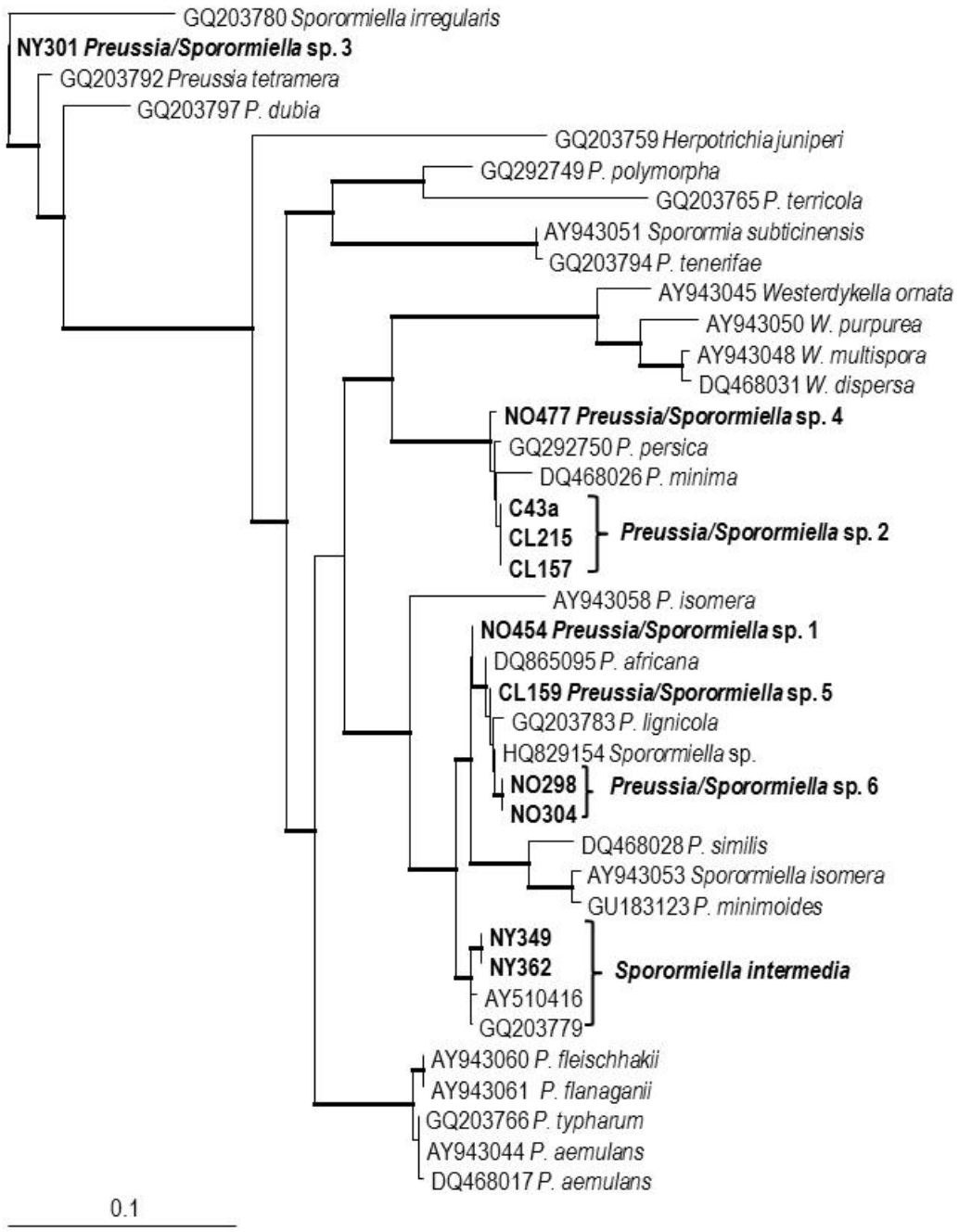


Figure 4. Maximum likelihood analysis of ITS sequences from Sporormiaceae species isolated in this study (CL) and detected from needles of five year old (NY) and eight year old (NO) trees and related accessions from the INSDs, rooted to *Preussia terricola*. LnL = -2384.06429, bar represents 2% expected variation. Note that *Preussia intermedia* is a synonym of *Sporormiella intermedia.* Branches in bold represent significance at P<0.01.

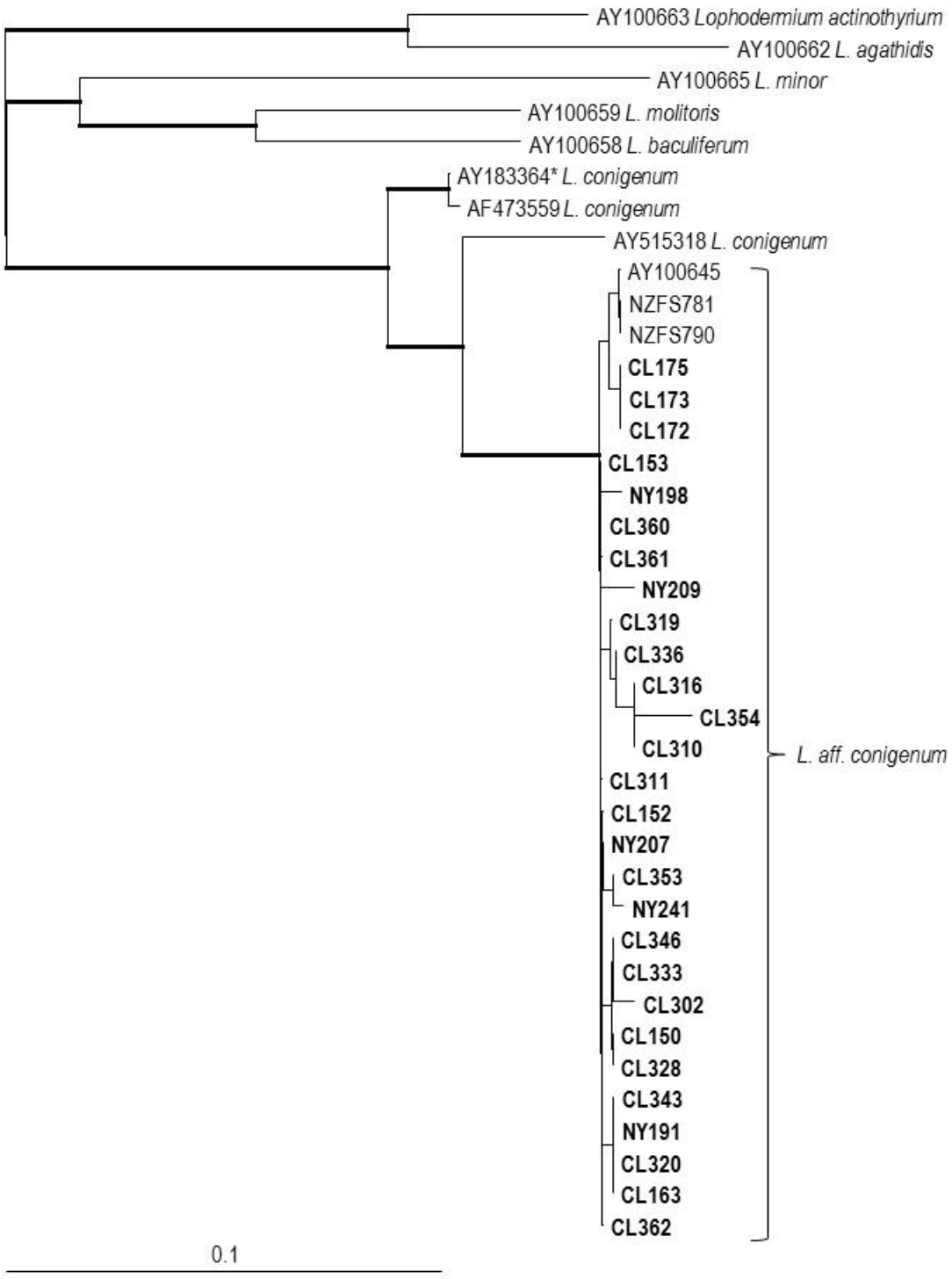


Figure 5. Maximum likelihood analysis of *Lophodermium conigenum* and *L. aff. conigenum* ITS sequences from cultures (CL and NZFS), amplified from needles of five year old trees (NY), and selected accession from the INSDs (supplementary material), rooted to *L. agathidis*. LnL = -2054.98553, bar represents 10% expected variation. Branches in bold represent significance at P<0.01.*AY183364 is from ATCC 28346 and is considered to represent authentic *L. conigenum.* NZFS790 and NZFS781 were also obtained for use as reference isolates of *L. conigenum,* but are included here as *L. aff. conigenum* as the degree of ITS sequence variation from the type isolate seems

uncharacteristically large for an ascomycete species and additional taxonomic studies may be required to adequately delineate the species.

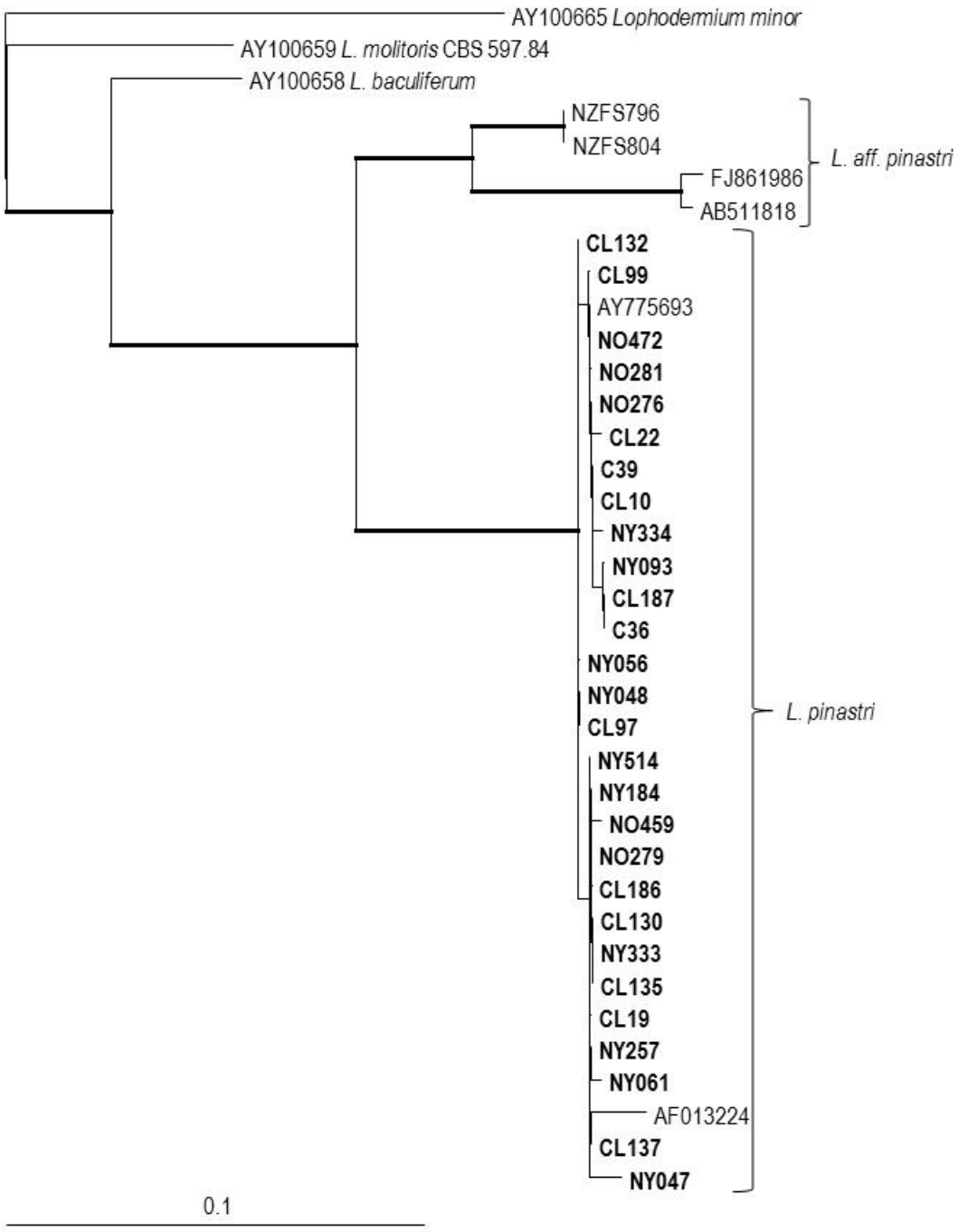


Figure 6. Maximum likelihood analysis of *L. pinastri* and *L. aff. pinastri* ITS sequences from cultures (CL and NZFS), needles of five year old (NY) and eight year old (NO) trees and selected accessions from the INSDs (supplementary material), rooted to *Lophodermium minor*. LnL = -1484.25756, bar represents 10% expected variation. Branches in bold represent significance at P<0.01. *AF013224 is from ATCC 28347 and is considered to represent authentic *L. pinastri.* NZFS796 and NZFS804 were also obtained for use as reference isolates of *L. pinastri*, but are included here as *L. aff. pinastri* as the degree of ITS sequence variation from the type isolate seems uncharacteristically large for an ascomycete species and additional taxonomic studies may be required to adequately delineate the species.

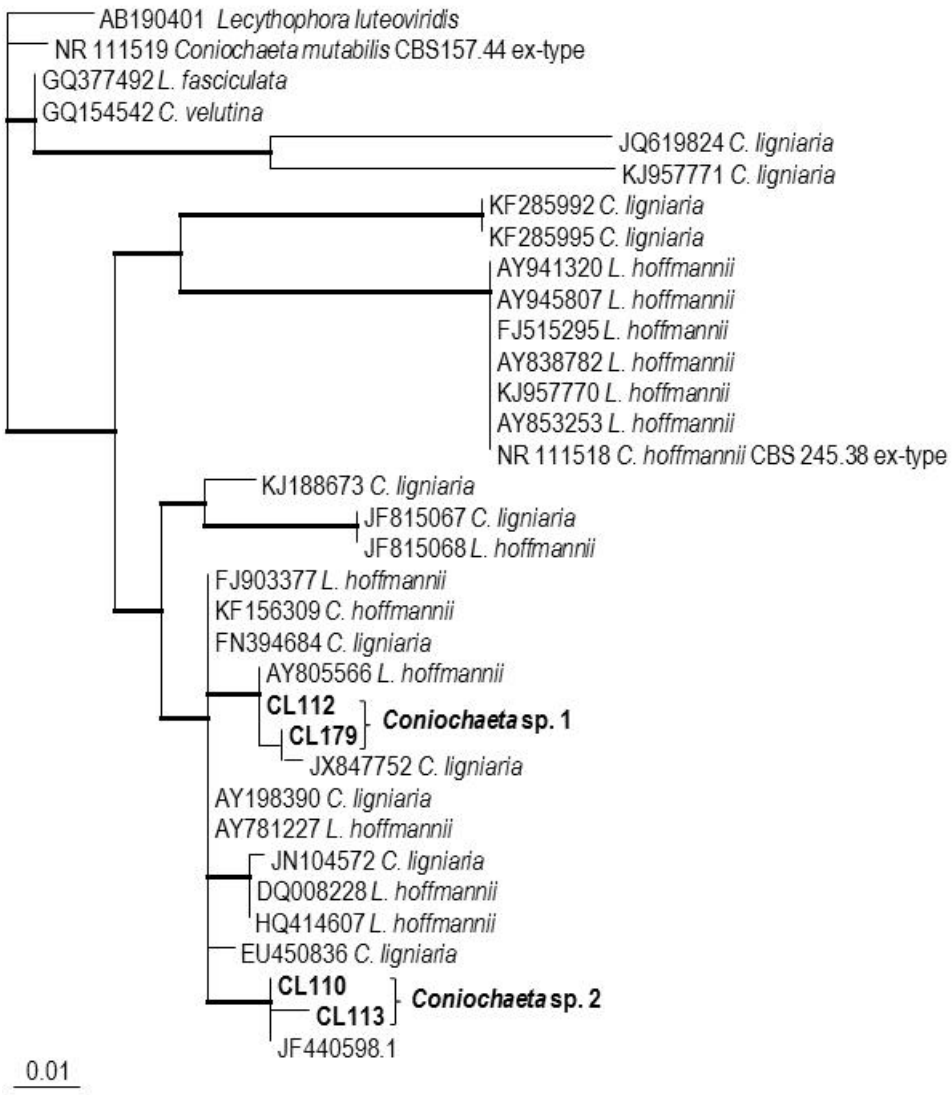


Figure 7: Maximum likelihood analysis of *Coniochaeta* ITS sequences from cultures (CL), and selected accessions from the INSDs, including all ITS sequences from the INSDs deposited as *C. ligniaria, C. hoffmannii* and *Lecythophora hoffmannii,* with the exception of JN942898, which was not alignable. LnL = -1170.03039, bar represents 1% expected variation. Branches in bold represent significance at P<0.01. Accessions are named as deposited, though all *Lecythophora* species have now been subsumed under *Coniochaeta.* NR_111518 is an ex-type isolate of *C. hoffmannii.*

Table 3.Number of OTUs and prevalence of the different fungal classes (and orders in the predominant classes) detected in this study, by isolation (CL = cultures) and PCR amplification followed by cloning.

Class/Order	No. of Orders	No. of Families	No. of MOTUs	Prevalence		
				N8*	N5**	CL (***)
Dothideomycetes	4	>11	73	36	33	21
Botryosphaeriales		1-3	3	2	3	0
Capnodiales		>3	47	35	31	0
Dothideales		>1	3	4	11	2
Pleosporales		>6	20	19	9	9
Eurotiomycetes	2	3	14	7	11	4
Lecanoromycetes	2	2	2	1	0	0
Leotiomycetes	>2	>6	17	29	24	119
Helotiales		>4	12	20	24	14
Rhytismatales		>1	3	14	7	99
Unknown		1-2	2	1	3	0
Pezizomycetes	1	2	3	0	0	7
Saccharomycetes	1	1	1	1	0	0
Sordariomycetes	4	>5	18	1	2	141
Hypocreales		1	1	0	0	1
Coniochaetales		1	2	0	0	77
Sordariales		>1	4	0	2	11
Xylariales		2	11	1	1	52
unknown Ascomycota	-	-	8	17	3	5
Tremellomycetes	2	3	9	3	5	0
Agaricomycetes	3	4	4	3	4	2
unknown Basidiomycota	-	-	4	0	4	3

*Occurrence of representatives from each fungal class in 36 pooled samples from 8 y.o. trees at a single site

**Occurrence of representatives from each fungal class in 34 pooled samples from 5 y.o. trees at 12 sites

***number of fungal isolates obtained from opportunistic sampling of 12 Tasmanian plantations

III.4. DISCUSSION

Fungal isolations were made in this study to provide support for the identification of MOTUs and to confirm their viability in pine needles. The sampling for isolations was opportunistic and not designed to allow a robust comparison of frequency of occurrence among sites or with the MOTUs detected by PCR and sequencing. Nonetheless, the substantial differences between the two datasets invite a comparison. Both techniques detected a few commonly occurring, widespread species and many more that were detected infrequently or from a limited geographic range. This is consistent with fungal endophyte communities in other *Pinus* species (Martinez-Alvarez et al. 2012, Peršoh 2013) other genera (Rajala et al. 2014) and indeed fungal communities in other substrates such as roots or soil (Danielsen et al. 2012). Most of the commonly isolated fungal species and many of the commonly detected MOTUs were consistent with previously known fungal associates of *P. radiata* or other pine or conifer species, e.g species of *Lophodermium, Cyclaneusma* and *Coniochaeta*. PCR and sequencing, however, revealed a number of common endophytes not previously recorded from *P. radiata.*

It is convenient to consider the fungal species in three groups; (1) those that were prominent and detected by both isolation and PCR, (2) those that were isolated frequently but not detected by PCR and sequencing, and (3) the frequently detected MOTUs that were

not represented by isolates. The first group consists of two species of *Lophodermium*, two of *Cyclaneusma,* and *Sydowia polyspora.*

Lophodermium pinastri was represented by 78 isolates and detected in 25 of the 70 bulk needle samples; *L.* aff. *conigenum* was less frequent, with 21 isolates and PCR detection from only four of the bulk needle samples. *Lophodermium pinastri* is well-known as an endophyte of several pine species and saprophytic in fallen needles (Hirose and Osono 2006, Johnston, Park, et al. 2003, Minter, Staley, et al. 1978) but has an unclear association with needlecast diseases. This lack of clarity may be due to the presence of a species complex within *Lophodermium pinastri* (Johnston, Park, et al. 2003) Figure 6) and further taxonomic studies may resolve this (Reignoux, Green, et al. 2014). The precise identity of the *L.* aff. *conigenum* isolates is also compromised by high intraspecific rDNA ITS sequence variation and high sequence similarity to *L. australe.* Our analysis (Figure 5) shows that isolates of both of these species are intermingled across three clades that differ by over 5% in rDNA ITS sequences. *L. conigenum* is considered to be saprophytic, but a study of fungal endophytes associated with healthy *P. radiata* trees and trees affected by needlecast in New Zealand (Ganley 2008) found *L. conigenum* only in trees affected by needlecast. Conclusions from this data would be premature, as the number of replications was low. Again, further taxonomic work is required to carefully delineate these two species. The Tasmanian isolates fall into the same clade as the reference isolates of *L. conigenum* from New Zealand, in contrast to *L. pinastri*, where the isolates from New Zealand are in different clades from the Tasmanian isolates. Recent work has uncovered several cryptic *Lophodermium* species in *P. sylvestris* (Reignoux, Green, et al. 2014) and a similar situation may exist in *L. conigenum*.

Cyclaneusma minus was recently shown to consist of two phylogenetic species (Prihatini, Glen, et al. 2014) and both were isolated and detected by PCR in this study,

though PCR detections outnumbered the isolations. This is likely due to the slow growth of *C. minus* in culture, allowing overgrowth by faster-growing fungal species. *C. minus* has been associated with needlecast in *P. radiata* (Choi and Simpson 1991, Podger and Wardlaw 1990) and is fairly widespread in Tasmania (Prihatini, Glen, et al. 2015b) but did not have a strong association with diseased trees in a family trial in Tasmania (Prihatini, Glen, et al. 2015a).

Sydowia polyspora was detected with moderate frequency and isolated twice. This species has been associated with current season needle necrosis (CSNN) in *Abies* spp. (Talgo et al. 2010) and needle yellowing and scorching in *Pinus halepensis* (Tinivella et al. 2014) however it is regarded as a normal component of needle mycota in many pines, with the ability to become pathogenic in stressed trees (Kraj 2009). It may be vectored by beetles (Jankowiak 2008). *S. polyspora* has been recorded from *P. radiata* in Kenya, Australia and New Zealand (Farr and Rossman 2014).

The second group contains three species that were isolated at moderate to high frequency, but not detected by PCR and DNA sequencing; *Coniochaeta* sp. 1 (73 isolates), Xylariaceae sp. 1 (19 isolates) and *Nemania diffusa* (13 isolates). Failure to detect these three species directly from DNA is puzzling; the rDNA ITS was readily amplified from mycelium of each of these species. It is possible that mismatches in the middle of the primer or towards the 5' end occur, which are not critical for amplification of a pure template, but result in preferential amplification of other species from a mixed template.

Coniochaeta ligniaria has been considered the teleomorph of *Lecythophora hoffmanni* (Damm, Fourie, et al. 2010) but (Khan, Gene, et al. 2013) transferred *L. hoffmanniii* to *Coniochaeta* as a distinct species, *C. hoffmannii.* Sequences of the rDNA ITS regions ascribed to *C. ligniaria, C hoffmannii* or *Lecythophora hoffmannii* are highly

variable (Figure 7), indicating either the existence of a species complex around *C. ligniara/C. hoffmannii,* or the frequent misapplication of these names to other species. A highly supported clade includes an ex-type isolate of *C. hoffmannii* as well as several clinical isolates (Chang et al. 2005), an isolate from sewage water in Belgium (FJ515295, unpublished) and an endophyte from Korean ginseng (Eo et al. 2014), but no isolates from conifers. This group has less than 95% sequence similarity to the isolates from pine needles. *C. ligniaria/L. hoffmannii* has been isolated from woody parts of several *Pinus* spp. (Lygis et al. 2010) but not previously recorded from pine needles. Our isolates fall into two distinct clades, one that clusters with *C. ligniaria* from woodland soil in the UK (JX847752, unpublished) and *L. hoffmannii* from *Picea abies* wood (Menkis, Allmer, et al. 2004) and the other group clusters with an isolate from *Pinus mugo* wood (JF440598, unpublished). As no sequence from type material of *C. ligniaria* is available, the exact identity of the isolates from pine needles is unclear though they are considered to belong to two distinct species of *Coniochaeta* on the basis of the phylogenetic analysis.

Identity of Xylariaceae sp. 1 is also unclear. It has high sequence similarity to a group of *Hypoxylon* spp., but *Hypoxylon* is polyphyletic (Kuhnert et al. 2014) and a secondary barcode is required for accurate identification (Suwannasai et al. 2013). Several species of *Hypoxylon* are known as endophytes (Bills et al. 2012, Ikeda et al. 2014). *Nemania diffusa* is endophytic in at least 19 plant species from 18 genera and 15 families (Okane et al. 2012), but also causes a soft rot decay of tea stems (Balasuriya and Adikaram 2009). This highlights the interdependence among environment, host and pathogen in producing disease. A similar example involves *Neonectria fuckeliana,* which is a weak pathogen or wound invader of *Picea* and *Abies* species in Europe but causes flute canker of *P. radiata* in New Zealand (Crane et al. 2009) and Chile (Morales 2009).

The third category included five MOTUs, all belonging to the Capnodiales, were detected in over 20% of the pooled needle samples but were not isolated in the current study. *Dothistroma septosporum* (Mycosphaerellaceae) is a well-known needlecast pathogen of *P. radiata* (de Wit, et al. 2012). It was detected mainly in older trees but did not appear to be correlated with high levels of needlecast (Prihatini et al. 2015a) and was present in few of the plantations studied (Prihatini et al. 2015b). Failure to isolate this species is most likely associated with its slow growth in culture and propensity to be overgrown by other fungal species. Recent studies of *D. septosporum* in *Abies* have relied on visual examination of needle symptoms, combined with DNA analyses, rather than isolation (Drenkhan, Adamson, et al. 2014).

Phaeotheca fissurella was also common at the more intensively sampled site (Prihatini, et al. 2015a) and present at three other sites (Prihatini et al. 2015b). This species is not well-known but has been recorded fruiting in *Pinus contorta* cankers caused by *Cronartium coleosporioides* (Sigler et al. 1981), suggesting it may be endophytic in that species.

The remaining MOTUs in this group are, like *Dothistroma* and *Phaeotheca*, all members of the Capnodiales, but could not be identified to species level because of low sequence similarity to isolates or to publicly available rDNA ITS sequences. All three have been identified as Teratosphaeriaceae spp., and another 27 MOTUs belonging to this family were detected, though at lower frequency.

A multitude of new species of Teratosphaeriaceae have been described recently, particularly from *Eucalyptus* hosts, but, to date, with the exception of *Dothistroma*, Teratosphaeriaceae on members of the Pinaceae have remained cryptic. Many fungal endophytes and biotrophic pathogens are difficult to isolate, slow-growing in culture (if at

all), and so easily overgrown by faster-growing, particularly saprophytic, fungi. Some may have particular nutritional requirements that were not met by the media used in the current study; biotrophs are prone to losing the ability to synthesize expensive nutrients such as thiamine (Spanu 2012). The failure to isolate *D. septosporum* highlights the difficulty of isolating members of this family. Teratosphaeriaceae sp. 23 was almost ubiquitous (Prihatini et al. 2015a, Prihatini et al. 2015b) and is most likely to be endophytic. Teratosphaeriaceae sp. 3, by contrast, was detected at only four sites, but was strongly associated with trees suffering from needlecast (Prihatini et al. 2015a) and young trees at sites that subsequently developed needlecast (Prihatini et al. 2015b). Teratosphaeriaceae sp. 26 has high sequence similarity to *Catenulostroma abietis*, and may be conspecific, but further taxonomic work is required to untangle this species complex (Crous et al. 2008). Kowalski and Zych (2002) isolated *Trimmatostroma cf. abietis* (*T. abietis* is a synonym of *C. abietis*) from symptomless shoots of *Pinus nigra.* (Gadgil and Dick 2009) isolated *T. abietis* from living needles of *P. muricata* and *P. radiata*, and stated that, while it was found in dead portions of living needles, it was unable to colonise living needles of *P. radiata* in pathogenicity tests.

Some *Teratosphaeria* species are aggressive pathogens of *Eucalyptus* spp. whereas others, such as *T. parva* and *T. associata*, occur commonly in a wide range of host species that includes *Protea* as well as *Eucalyptus* species and both may represent species complexes (Hunter, et al. 2011). MOTUs with high sequence similarity to these two species were detected at low frequency in pine needles, as were *T.* aff. *suttonii* and *T.* aff. *capensis.* None of the eucalypt pathogens known to be common in Tasmania, *T. cryptica, T. nubilosa* and *T. molleriana* (Milgate et al. 2001), were detected from pine needles, supporting the hypothesis that the fungi detected were colonizing rather than superficially contaminating the pine needles.

No clear conclusions can be drawn regarding any of the fungi detected in this study and their relationship to tree health, though some interesting avenues for further inquiry have been indicated. It is clear from this study and many others (Balasuriya and Adikaram 2009, Crane et al. 2009) that a knowledge of the role a fungal species plays in one host and location is not necessarily a confident predictor of its effect in another host species or in a different environment. Many of the species detected in this study have been linked to needle diseases of conifers and some have been highlighted as potentially playing a role in spring needlecast in Tasmania, but more work is required to ascertain which organism(s) may have a direct causal role. More comprehensive metagenomic studies using next-generation sequencing will develop our understanding of fungal endophytes, their lifestyles and host ranges. Targeted attempts to isolate and describe previously unknown members of the Teratosphaeriaceae from pine needles would increase understanding of their roles in pines and other plants.

III.5 SUPPLEMENTARY MATERIAL

Table S1. Morphological grouping of isolates

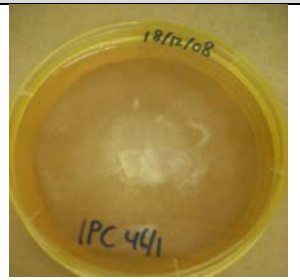
Table S2. Accession codes for all sequences generated in this study

Figure S1. Maximum likelihood analysis of all *L. conigenum* and *L. australe* ITS sequences from the INSDs.

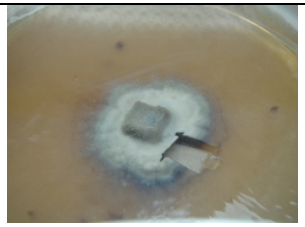
Figure S2. Maximum likelihood analysis of *L. pinastri* ITS sequences from the INSDs.

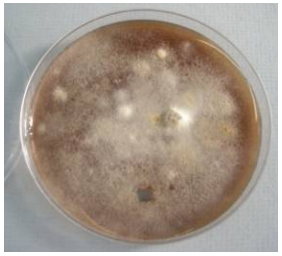
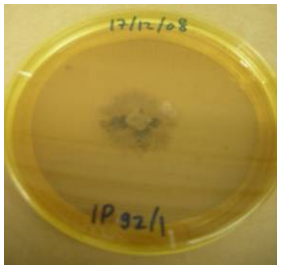
Table S1. Grouping of the most common cultures according to macroscopic morphological characters (Stalpers, 1978). Growth rates were estimated by measuring colony diameter at 1-month-old: growth was classified as slow, medium and fast when the diameter of cultures were < 2 cm, 2-4 cm, > 4 cm, respectively. The OTU identifications and phylogenetic analyses are listed in supplements Table S2 and Figures S1 and S2.

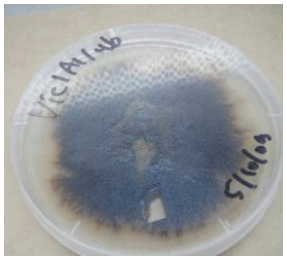
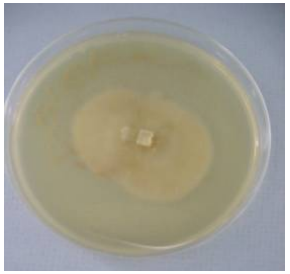
Group	Gross morphology	No. of sequenced isolates	OTU identification	
1	Orange mycelium, zonate texture, irregular margin and slow to medium growth rate.	77	*Coniochaeta* spp. (comprised of 2 OTUs)	
2	White mycelium, felt texture, circular and medium growth rate.	9	*Fimetariella rabenhorstii*	

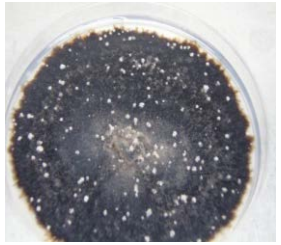
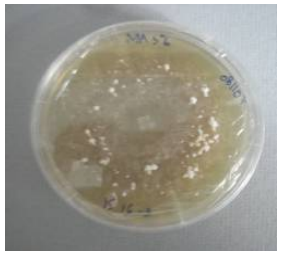
Group	Gross morphology	No. of sequenced isolates	OTU identification	
3	White mycelium, silky texture, circular and fast growth rate.	43	*Lophodermium pinastri*	
4	White or yellowish mycelium, irregular margin, white or black crustose and slow growth rate	35	*L.pinastri*	
5	White mycelium, finely floccose texture, slightly irregular margin and fast growth rate.	12	*L.* aff*. conigenum*	

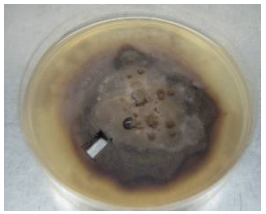
Group	Gross morphology	No. of sequenced isolates	OTU identification	
6	White mycelium, brown reverse, thick woolly texture, irregular margin, medium growth rate.	9	*L.* aff. *conigenum*	
7	White mycelium, silky texture, circular with radial streaks of more cottony mycelium and fast growth rate.	20	Xylariaceae spp. (comprised of 4 OTUs)	
8	White mycelium brown reverse, thick silky texture, farinaceous patches, circular and medium to fast growth rate.	21	Xylariales spp. (comprised of 3 OTUs)	

Group	Gross morphology	No. of sequenced isolates	OTU identification	
9	White or pinkish mycelium, thick silky texture with pink tinge, crustose central patches, irregular margin and medium growth rate.	3	*C. minus* ' verum'	
10	White mycelium with brown reverse, velvety texture, some with white crustose central patches, irregular margins and slow to medium growth rate.	2	*C. minus* ' simile'	
11	White mycelium with brown reverse, felty surface, irregular margins and medium growth rate	2	*Mollisia* sp.	

Group	Gross morphology	No. of sequenced isolates	OTU identification	
12	White mycelium, woolly floccose texture, irregular margins and medium growth rate.	2	*Phoma* sp. 1	
13	Light brown mycelium with brown reverse, circular or regular margin, felt texture and fast growth rate.	5	*Phoma* sp. 1	
14	White orange or yellowish mycelium, silky or felty texture, irregular margin, medium growth rate.	4	*Sporormiella* spp. (comprised of 2 OTUs)	

Group	Gross morphology	No. of sequenced isolates	OTU identification	
15	White mycelium to blackish mycelium, thick, felty surface, irregular margin and medium growth rate.	4	*Sydowia polyspora*	
16	White to light brown mycelium, brown reverse, irregular margin and medium growth rate.	7	Helotiales spp. (3OTUs)	
17	White greyish mycelium, black reverse, silky texture, circular with radial streak, farinaceous black central patches, medium to fast growth rate.	3	*Biscogniauxia* sp.	

Group	Gross morphology	No. of sequenced isolates	OTU identification	
18	White greyish mycelium, felt, zonate texture, irregular margin and slow growth rate.	2	*Lasiosphaeria lanuginosa*	
19	Black mycelium, granular, with white pycnidia, slightly irregular margin and fast growth rate.	2	*Strasseria geniculata*	
20	White mycelium, thick silky texture, floccose patches, irregular margin, medium to fast growth rate.	2	*Chromelosporium carneum*	

Group	Gross morphology	No. of sequenced isolates	OTU identification	
21	White mycelium, lacunose texture, circular and fast growth rate.	3	*Kendrickiella phycomycoides*	
22	Brown mycelium with dark brown reverse, farinaceous (powdery) texture, irregular margin and medium growth rate.	3	*Lophiostoma* spp. (3 OTUs)	
23	White to black mycelium, felty texture, irregular margin, medium growth rate.	2	*Aureobasidium pullulans*	

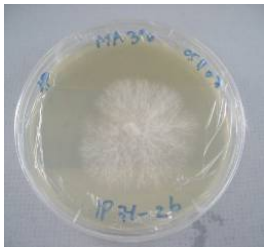
Group	Gross morphology	No. of sequenced isolates	OTU identification	
24	White mycelium, thick cottony texture, radial patches irregular margin, medium to fast growth rate.	3	Pezizales sp. (3 OTUs)	
25	Transparent or black mycelium, felty, zonate texture, irregular margin and medium growth rate.	1	*Ceuthospora pinastri*	
26	White-greyish mycelium with brown reverse, felty texture, irregular margin and medium growth rate.	1	Basidiomycete sp. 5	

Table S2. Accession codes for all sequences generated in this study.

Sequence Sample ID	Accession codes
NY049	KJ406757
NY050	KJ406758
NY058	KJ406759
NY063	KJ406760
NY096	KJ406761
NO181	KJ406762
NY249	KJ406763
NY252	KJ406764
NY261	KJ406765
NY352	KJ406766
NY365	KJ406767
NY368	KJ406768
NY405	KJ406769
NY466	KJ406770
NY468	KJ406771
NO317	KJ406772
NO324	KJ406773
NO13	KJ406774
NO16	KJ406775
NO75	KJ406776
NO93	KJ406777
NO132a	KJ406778
NO188	KJ406779
NO199b	KJ406780
NO217b	KJ406781
NO361	KJ406782
NO511	KJ406783

Sequence Sample ID	Accession codes
NY001	KJ406784
NY013	KJ406785
NY018	KJ406786
NY025	KJ406787
NY030	KJ406788
NO357	KJ406789
NO409	KJ406790
NY218	KJ406791
NY321	KJ406792
NY324	KJ406793
NO219	KJ406794
NY080	KJ406795
NO197a	KJ406796
NO235a	KJ406797
NO405	KJ406798
NO428	KJ406799
NO431	KJ406800
NY022	KJ406801
NY097	KJ406802
NY431	KJ406803
NO24	KJ406804
NO042	KJ406805
NO099	KJ406806
NO096	KJ406807
NO024	KJ406808
NO087	KJ406809
NO116	KJ406810
NO137	KJ406811

Sequence Sample ID	Accession codes
NO173	KJ406812
NO200	KJ406813
NO211	KJ406814
NO236	KJ406815
NO411	KJ406816
NO417	KJ406817
NO476	KJ406818
NO118b	KJ406819
NO008b	KJ406820
NO027b	KJ406821
NO335b	KJ406822
NO370	KJ406823
NY106	KJ406824
NY124	KJ406825
NY104	KJ406826
NY420	KJ406827
NY426	KJ406828
NY491	KJ406829
NO247	KJ406830
NY217	KJ406831
NY210	KJ406832
NO260	KJ406833
NO245	KJ406834
NO250	KJ406835
NY394	KJ406836
NY101	KJ406837
NY110	KJ406838
NY354	KJ406839

Sequence Sample ID	Accession codes
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NO534	KJ406841
NY356	KJ406842
NO406	KJ406843
NO482	KJ406844
NY517	KJ406845
NY509	KJ406846
NY518	KJ406847
NY524	KJ406848
NY248	KJ406849
NY255	KJ406850
NY256	KJ406851
NO394	KJ406852
NO178	KJ406853
NO237	KJ406854
NO241	KJ406855
NO68	KJ406856
NO72	KJ406857
NO142	KJ406858
NO190	KJ406859
NO192	KJ406860
NO234	KJ406861
NY362	KJ406862
NY349	KJ406863
NO304b	KJ406864
NO298b	KJ406865
NO477	KJ406866
NY301b	KJ406867

Sequence Sample ID	Accession codes
NO454	KJ406868
NY211	KJ406869
NY214	KJ406870
NY216	KJ406871
NY392	KJ406872
NY398	KJ406873
NY399	KJ406874
NO462	KJ406875
NY245b	KJ406876
NY247	KJ406877
NY235	KJ406878
NY250	KJ406879
NY242b	KJ406880
NO170	KJ406881
NO174	KJ406882
NO521	KJ406883
NY516	KJ406884
NO531	KJ406885
NO529	KJ406886
NY318	KJ406887
NY319	KJ406888
NY320	KJ406889
NY384	KJ406890
NY386	KJ406891
NY502	KJ406892
NY506	KJ406893
NY520b	KJ406894
NY522b	KJ406895

Sequence Sample ID	Accession codes
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NO396	KJ406897
NO438b	KJ406898
NO451	KJ406899
NO489	KJ406900
NO510	KJ406901
NY174	KJ406902
NY166	KJ406903
NY178	KJ406904
NY044	KJ406905
NY133	KJ406906
NY135	KJ406907
NO450	KJ406908
NO277	KJ406909
NY004	KJ406910
NY486	KJ406911
NO139	KJ406912
NO052	KJ406913
NY479	KJ406914
NY483	KJ406915
NY484	KJ406916
NY490	KJ406917
NY489	KJ406918
NO347	KJ406919
NO275	KJ406920
NY224	KJ406921
NY230	KJ406922
NY234	KJ406923

Sequence Sample ID	Accession codes
NY138	KJ406924
NY199	KJ406925
NY220	KJ406926
NY223	KJ406927
NY225	KJ406928
NY231	KJ406929
NY232	KJ406930
NY226	KJ406931
NO103	KJ406932
NO331	KJ406933
NO384	KJ406934
NO386	KJ406935
NO421	KJ406936
NO425	KJ406937
NO426	KJ406938
NO427	KJ406939
NO429	KJ406940
NO432	KJ406941
NO437	KJ406942
NO471	KJ406943
NY191	KJ406944
NY198	KJ406945
NY207	KJ406946
NY209	KJ406947
NY241	KJ406948
NY047	KJ406949
NY093	KJ406950
NY334	KJ406951

Sequence Sample ID	Accession codes
NO276	KJ406952
NO281	KJ406953
NO472	KJ406954
NY048	KJ406955
NY056	KJ406956
NY061	KJ406957
NY184	KJ406958
NY257	KJ406959
NY333	KJ406960
NY514	KJ406961
NO279	KJ406962
NO459	KJ406963
NY397	KJ406964
NY401	KJ406965
NY192	KJ406966
NY477	KJ406967
NY480	KJ406968
NY482	KJ406969
NY487	KJ406970
NO487	KJ406971
NO488	KJ406972
NO369b	KJ406973
NO366b	KJ406974
NO368b	KJ406975
NO498b	KJ406976
NO327b	KJ406977
NO336b	KJ406978
NO372b	KJ406979

Sequence Sample ID	Accession codes
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NY079	KJ406981
NY186	KJ406982
NY183	KJ406983
CL341	KJ406984
CL51	KJ406985
CL349	KJ406986
CL167	KJ406987
CL340	KJ406988
CL342	KJ406989
CL151	KJ406990
CL199	KJ406991
CL213	KJ406992
C14	KJ406993
CL208	KJ406994
CL166	KJ406995
CL168	KJ406996
CL177	KJ406997
CL184	KJ406998
CL121	KJ406999
CL116	KJ407000
C12	KJ407001
CL305	KJ407002
CL6	KJ407003
CL326	KJ407004
CL335	KJ407005
CL156	KJ407006
CL176	KJ407007

Sequence Sample ID	Accession codes
CL308	KJ407008
CL180	KJ407009
CL181	KJ407010
CL306	KJ407011
CL350	KJ407012
CL315	KJ407013
CL159	KJ407014
CL157	KJ407015
CL215	KJ407016
C43a	KJ407017
C10	KJ407018
C13	KJ407019
CL313	KJ407020
CL325	KJ407021
CL338	KJ407022
CL149	KJ407023
CL362	KJ407024
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CL172	KJ407029
CL173	KJ407030
CL175	KJ407031
CL302	KJ407032
CL310	KJ407033
CL311	KJ407034
CL319	KJ407035

Sequence Sample ID	Accession codes
CL320	KJ407036
CL328	KJ407037
CL333	KJ407038
CL336	KJ407039
CL343	KJ407040
CL346	KJ407041
CL353	KJ407042
CL360	KJ407043
CL187	KJ407044
CL10	KJ407045
CL22	KJ407046
CL99	KJ407047
CL97	KJ407048
CL132	KJ407049
CL19	KJ407050
CL130	KJ407051
CL135	KJ407052
CL137	KJ407053
CL186	KJ407054
CL122	KJ407055
CL307	KJ407056
CL123	KJ407057
CL309	KJ407058
C38	KJ407059
CL207	KJ407060
CL218	KJ407061
IP15	KJ407062
CL205	KJ407063

Sequence Sample ID	Accession codes
CL160	KJ407064
CL357	KJ407065
CL110	KJ407066
CL112	KJ407067
CL113	KJ407068
CL179	KJ407069
CL145	KJ407070
CL170	KJ407071

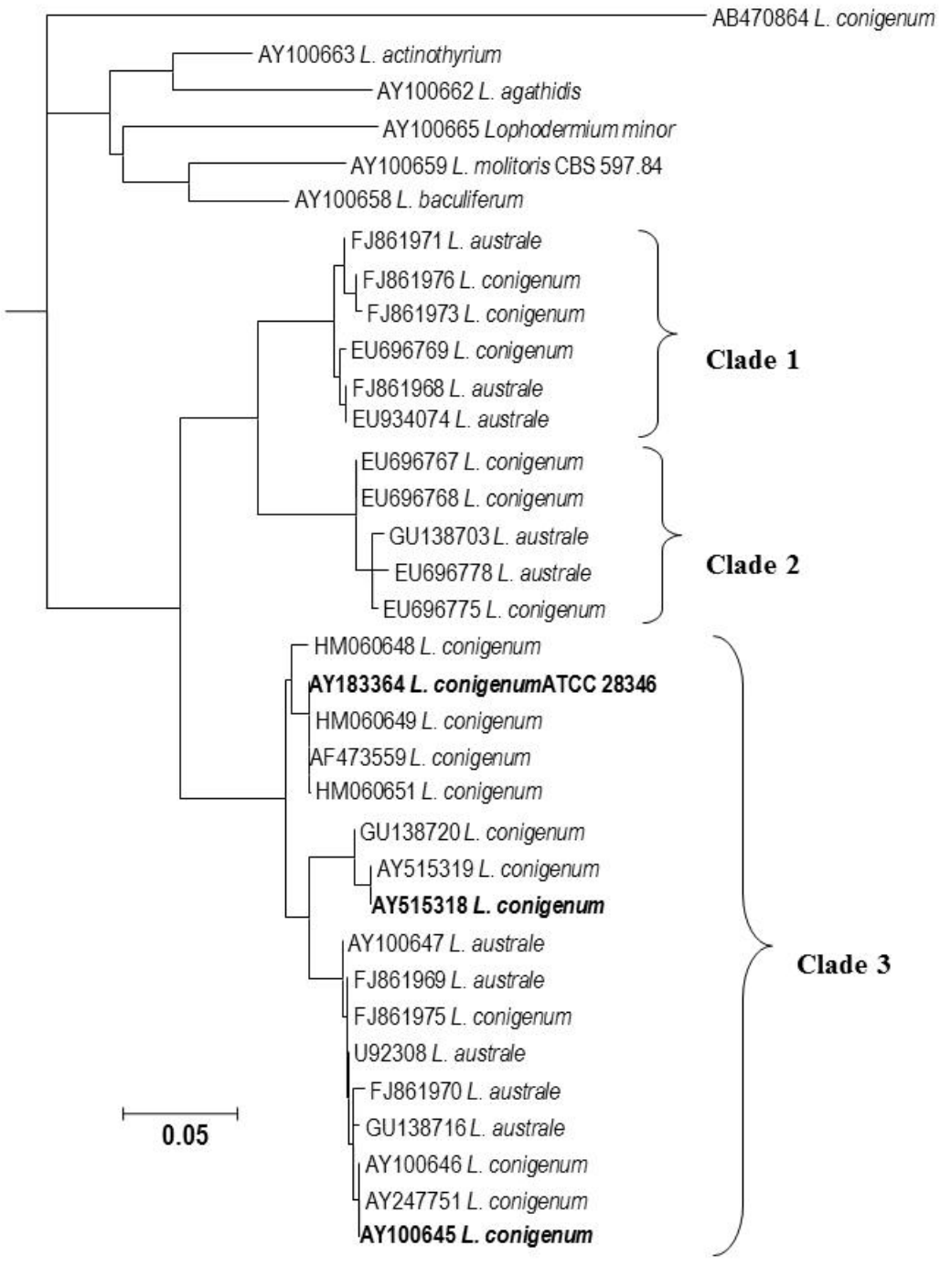

Figure S1. Maximum likelihood analysis of all *L. conigenum* and *L australe* ITS sequences from the INSDs. Clade 3 includes an ex-type culture and is considered to represent reliably identified isolates of *L. conigenum*, though up to 3% variability is seen in this clade, and accession numbers in bold are used as reference sequences in further analysis (Figure 12). Bar represents 5% expected variation.

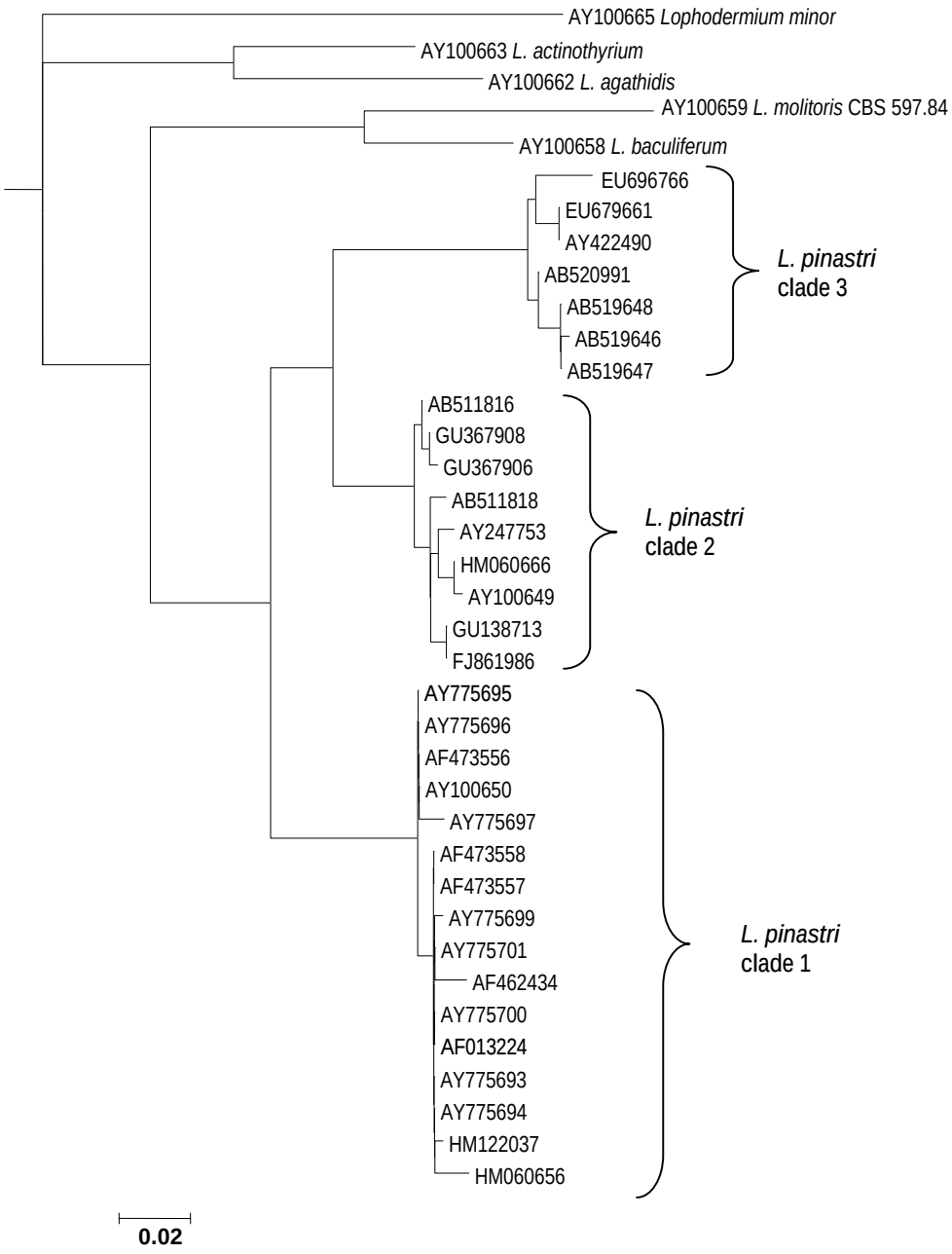

Figure S2. Maximum likelihood analysis of *L. pinastri* ITS sequences from the INSDs. AF013224, though not linked to a publication, represents the ex-type isolate ATCC 28347, therefore Clade 1 is considered to represent reliably identified isolates of *L. pinastri*, and accession numbers in bold were used as reference sequences in further analysis (Figure 13). Bar represents 2% expected variation.

IV. MULTIGENE PHYLOGENETIC STUDY OF *CYCLANEUSMA* SPECIES

Abstract

Cyclaneusma Needle Cast is associated with the fungal pathogen *Cyclaneusma minus*. The disease has led to significant losses of *Pinus radiata* production in New Zealand, but the disease appears less serious in Australia. Two cultural morphotypes of *C. minus* have been discriminated in New Zealand, and isolates from Australia also correspond to these two morphotypes. To examine if the two morphotypes of *Cyclaneusma* are different species, phylogenetic analysis using sequences from five gene regions (rDNA ITS, mt-LSU, n-LSU, tef–1 and bT-1) was conducted. This study revealed that *C. minus* consists of two distinct clades which correspond to the two morphotypes. One morphotype ('verum') was more closely related to *Cyclaneusma niveum* than to the other morphotype ('simile'). A morphological description is required to formalise the new species represented by the 'simile' clade; an investigation of the individual pathogenic behaviours of both morphotypes is also required.

IV.1. INTRODUCTION

The unambiguous characterisation of fungal pathogens underpins the development of successful biosecurity and disease management strategies. Firstly, the correct identification of an incursion can facilitate an appropriate and rapid response (Carnegie and Cooper 2011; McDougal, Schwelm et al. 2011; Fichtner, Rizzo et al. 2012). Secondly, the study of inter- and intraspecific responses to environmental stimuli such as changing climatic conditions (Fabre et al. 2012; Sturrock 2012; Tellenbach and Sieber 2012) may assist in predicting the likely disease impacts. Thirdly, selection for and deployment of host resistance, chemical control or putative biocontrol agents such as endophytes, also requires a sound knowledge of the pathogen (Ganley et al. 2008; Graça et al. 2011).

The genus Cyclaneusma was erected by Dicosmo et al. (1983) to accommodate Naemacyclus minor and N. niveus which have ascospores and hymenium with quite different characteristics to the type species of that genus; they also renamed the type species from Naemacyclus pinastri to N. fimbriatus. Two different species, C. minus and C. niveum are recognised in Cyclaneusma (Dicosmo et al. 1983) and both have been recorded in many different species of Pinus (Botella and Diez 2011; Farr and Rossman 2013; Sieber et al. 1999). Studies of Cyclaneusma needle diseases indicate that C. minus is more pathogenic than C. niveum; the latter is considered a weak pathogen (Bulman and Gadgil 2001; Kistler and Merril 1978; Petrini and Petrini 1985).

Cyclaneusma minus is found in Asia, Australia (Choi and Simpson 1991), New Zealand (Bulman 1988), Europe, e.g. Spain (Zamora et al. 2008), USA (Merrill and Wenner 1996), Chile (USDA Forest Service 1993) and South Africa (Botes et al. 1997). In New Zealand, *C. minus* is considered to be a serious threat to 11-20 year old *P. radiata* plantations (Bulman 1988; Bulman and Gadgil 2001) but in Australia, although ubiquitous, *C. minus* has been historically regarded as less virulent (Choi and Simpson 1991; Stahl 1966). In Tasmania it is putatively involved in Spring Needle Cast as an opportunist (Podger and Wardlaw 1990). Spring Needle Cast is not considered to be a classical needle blight disease caused by a primary fungal pathogen, but by a suite of endophytic fungi (*C. minus*, *Lophodermium pinastri* and *Strasseria geniculata*) that are triggered into secondary pathogenic activity by an environmental stress. It is observed after canopy closure when trees are 7 years old (Podger and Wardlaw 1990).

Variation in morphological characters of *C. minus* cultures (as *Naemacyclus minor)* was initially reported in Serbia by Karadzic (1981); variation of cultural morphology between populations has also been reported in New Zealand (Beets et al. 1997; Bulman and Gadgil 2001). Differences in mycelium colour, colony texture, pigmentation, sporulation and growth *in vitro* have been used to separate *C. minus* into two different morphotypes viz *C. minus* 'verum' and *C. minus* 'simile' (Bulman and Gadgil 2001). A study of genetic variation in *C. minus* cultures from New South Wales (Australia) and New Zealand using RAPD, SSR and IGS molecular markers revealed two main groups in *C. minus* that corresponded to the grouping by morphological characters, though the level of similarity in rDNA ITS sequences (~95%) indicated that they were closely related (Crowley 2006). Given this high level of morphological and genetic variation, we hypothesised that *C. minus* could be divided into two distinct phylogenetic species. As RAPD and SSR genotyping are insufficient as a basis for delineation of novel species because results are often difficult to reproduce (Voigt and Wostemeyer 1995), a multigene phylogenetic study was conducted to test this hypothesis based on isolates from Tasmania, mainland Australia, New Zealand and Europe.

Phylogenetic studies, based on DNA sequences, have been useful in resolving taxonomic confusion in forest pathogens, as in the genera *Mycosphaerella* (Cheewangkoon et al. 2009) and *Melampsora* (Vialle et al. 2013). Phylogenetic species recognition is rapidly becoming the preferred method for discriminating cryptic fungal species (Taylor et al. 2000). Increasingly, phylogenetic studies have been based on multiple gene regions to increase confidence that the reconstructed tree represents a species rather than a gene phylogeny (Cortinas et al. 2006; Douhan et al. 2008, Queloz et al. 2011; Vialle et al. 2013). Multigene phylogenetic analysis of *Dothisthroma septosporum*, the cause of red-

band needle blight in *Pinus* species including *P. radiata*, identified two distinct species associated with the disease, *D. pini* and *D. septosporum*, that had been formerly classed as a single species (Barnes et al. 2004). Similarly, cryptic speciation was identified in *Hymenoscyphus albidus* and the new species *H. pseudoalbidus* was identified as the likely causal agent of Ash dieback (Queloz et al. 2011). Multigene phylogenetic studies have also been used to discriminate *Coniothyrium zuluense* from South Africa and *C. gauchensis* from Argentina and Uruguay, two distinct species that produce identical stem canker symptoms in *Eucalyptus* (Cortinas et al. 2006). Phylogenetic species recognition based on a multigene phylogeny was used in this study to investigate the existence of a genetically distinct, cryptic *Cyclaneusma* species in Australia and New Zealand.

IV.2. METHODS AND METHODS

IV.2.1. Isolates

Isolates of *Cyclaneusma* from Australia, New Zealand, Kenya, Germany, France and Italy were examined by DNA sequencing (Table 1). Those from Australia and New Zealand included isolates of both morphotypes. Isolates of other species belonging to the orders *Helotiales* (*Ceuthospora* sp.) and *Rhytismatales* (*Lophodermium* species) were also included as outgroups in the phylogenetic analyses.

IV.2.2. DNA extraction and PCR amplicfication

DNA from all 47 isolates was extracted using an SDS extraction buffer and purified by a silica-binding method according to Glen et al. (2002). Prior to PCR amplification, DNA was diluted 1/100 in TE buffer (10mM Tris-Cl, pH 8.0, 1mM EDTA).

Table 1. Isolates of *Cyclaneusma* species and other species of *Helotiales* and *Rhytismatales* included in the phylogenetic analyses.

Species	Cultural morphotype*	Isolate code	Source^	Origin#
Cyclaneusma niveum		N214 = CBS 495.73 (Neotype)	BBA	Germany
C. niveum		N215	BBA	Italy
C. niveum		N218	BBA	France
C. niveum		N219	BBA	Germany
C. minus	nd	DFR1224-6	CSIRO	ACT, Australia
C. minus	nd	DFR1224-10	CSIRO	ACT, Australia
C. minus	nd	DFR 1226-1	CSIRO	NSW, Australia
C. minus	nd	DFR 1226-5	CSIRO	NSW, Australia
C. minus	nd	DFR 1227-1	CSIRO	NSW, Australia
C. minus	nd	DFR 1227-4	CSIRO	NSW, Australia
C. minus	nd	DFR 1228-5	CSIRO	NSW, Australia
C. minus	nd	DFR 1228-9	CSIRO	NSW, Australia
C. minus	verum	1062	Scion	New Zealand
C. minus	verum	MC303L/1	Scion	New Zealand
C. minus	simile	MC103y/3	Scion	New Zealand
C. minus	simile	MC103y/5	Scion	New Zealand
C. minus	verum	MC303L/6B	Scion	New Zealand
C. minus	simile	300L/2c	Scion	New Zealand
C. minus	simile	107/7	Scion	New Zealand
C. minus	simile	104/5	Scion	New Zealand
C. minus	simile	102g/4	Scion	New Zealand
C. minus	simile	104/4	Scion	New Zealand
C. minus	simile	101y/4	Scion	New Zealand
C. minus	verum	303L/3c	Scion	New Zealand
C. minus	verum	318	Scion	New Zealand
C. minus	simile	317B	Scion	New Zealand
C. minus	simile	301H/3c	Scion	New Zealand
C. minus	nd	N226	BBA	Germany
C. minus	nd	N231	BBA	Kenya
C. minus	nd	C348	BBA	Germany
C. minus	verum	SN8/5	TIA	Tas, Australia
C. minus	verum	O07-2	TIA	Tas, Australia
C. minus	simile	O08/1	TIA	Tas, Australia
C. minus	verum	V1A2/2b	TIA	Vic, Australia
C. minus	verum	V3B/3b	TIA	Vic, Australia
C. minus	nd	PN0362	CSIRO	SA, Australia
C. minus	nd	PN0363	CSIRO	SA, Australia
Lophodermium. sp.		NZFS796	Scion	New Zealand
Lophodermium. sp.		NZFS804	Scion	New Zealand
L. molitoris		NZFS789	Scion	New Zealand
L. conigenum		NZFS790	Scion	New Zealand
L. conigenum		NZFS781	Scion	New Zealand
L. pinastri		O7/2C	TIA	Tas, Australia
L. pinastri		O7/3	TIA	Tas, Australia
Ceuthospora sp.		NZFS504	Scion	New Zealand
Strasseria geniculata		NZFS506	Scion	New Zealand
S. geniculata		MC7964/2	Scion	New Zealand

* Morphotype (if determined) of *C. minus* cultures, nd = not determined.
^BBA - Biologische Bundesanstalt für Land-und Forstwirtschaft, Germany
CSIRO - Commonwealth Scientific and Industrial Research Organisation, Australia
Scion – A Crown Research Institute, New Zealand
TIA- Tasmanian Institute of Agriculture, isolated during this study, these cultures will be deposited with the International Collection of Micro-organisms from Plants, New Zealand.
[#]Australian state abbreviations: ACT = Australian Capital Territory; NSW = New South Wales; Tas = Tasmania; Vic = Victoria; SA = South Australia

Five different primer pairs (Table 2) were used to amplify the rDNA ITS, and fragments of the mitochondrial rDNA LSU (mt-LSU), nuclear rDNA LSU (n-LSU), translation elongation factor 1α (*tef-1*) and β-tubulin 1 (βT-1) genes. PCR reactions had a final concentration of ingredients as follows: 67mM Tris-HCl, pH 8.8; 16mM $(NH_4)_2SO_4$ (in 10× NH_4-based reaction buffer supplied by Bioline, UK); 2.0 mM magnesium chloride (Promega, USA); 200 μM each deoxynucleotide triphosphate (Bioline, UK); 0.25 μM each oligonucleotide primer (Geneworks, Australia); 0.02 units/μL of Mangotaq DNA polymerase (Bioline, UK); 0.2 μg/μL of bovine serum albumin (Fisher Biotec, Australia) to reduce enzyme inhibition that may be present in the DNA template (Kreader 1996); 10 μL of the diluted DNA as template and water for injection (Astra Zeneca, Australia) to make volume up to 50 μL. Amplification was performed using a Peltier Thermal Cycler PTC-225 (MJ Research) and the following temperature profile: 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C (or 50°C) for 30 s and 72°C for 2 min, with a final 7 min extension at 72°C (Table 2).

Table 2. Primers used in this study and the annealing temperature (TA) used for each primer pair.

Gene region	Primer name	Sequence (5'-3')	TA (°C)	Reference
Translation elongation factor 1-α	EF1-728F	CAT CGA GAA GTT CGA GAA GG	55	Carbone et al. 1995
	EF1-986R	TAC TTG AAG GAA CCC TTA CC		
β-tubulin 1	Bt1a	TTC CCC CGT CTC CAC TTC TTC ATG	50	Glass and Donaldson 1995
	Bt1b	GAC GAG ATC GTT CAT GTT GAA CTC		
rDNA ITS	ITS4	TCC TCC GCT TAT TGA TAT GC	55	White et al. 1990
	ITS1-F	CTT GGT CAT TTA GAG GAA GTA A		Gardes and Bruns 1993
mt-LSU	ML3	GCT GGT TTT CTA CGA AAC ATA TTT AAG	55	White et al. 1990
	ML4	GAG GAT AAT TTG CCG AGT TCC		
n-LSU	NL5mun	GCA TAT CAA TAA GCG GAG GA	50	Egger 1995
	NL4	GGT CCG TGT TTC AAG ACG G		O'Donnell 1993

Prior to DNA sequencing, PCR products were separated by gel electrophoresis on a 1% agarose gel (Fisher Biotech, Australia) at 10 volts/cm for 30 min using MI-DEAR 120 High Performance Gel System (Biokeystone, USA). Gels were visualized on a Vilber Lourmat transilluminator after staining with ethidium bromide 0.5 µg/mL (MOBIO, USA) for 20 min and images captured using a Vilber Lourmat camera (Marne-la-Vallée, France).

IV.2.4. DNA Sequencing analysis and phylogenetic analysis

PCR products were sequenced by Macrogen (Korea). Chromatograms were viewed in ChromasPro ver. 1.34 (Technelysium, Brisbane, Australia) and trimmed for quality at each end and then the sequence was saved in FASTA format. All sequences were submitted to GenBank: accession numbers KF013542-KF013588 (ITS); KF013589-KF013615 (mt-LSU); KF013626-KF013670 (n-LSU); (KF356553-KF356591) (βT-1); (KF356592-KF356636) (*tef-1*). All available sequences from each gene region were aligned using

CLUSTALW (Thompson et al. 1994). Alignments of these five gene regions were edited in Bioedit (Hall 1999) before phylogenetic analysis. Polymorphisms were verified against the chromatograms and alignments adjusted where necessary. Gaps longer than one nucleotide were treated as missing data.

Phylogenetic trees were produced for each gene region by the Maximum Likelihood and Maximum Parsimony methods, sequences from *Lophodermium species* (*Rhytismatales*), *Ceuthospora* sp. (*Helotiales*) and *Strasseria geniculata* (incertae sedis) were included as outgroups in the phylogenetic analyses. Trees were constructed using DNAML and DNApars from the PHYLIP package (Felsenstein, 1981). A Bayesian analysis was performed using MrBayes (Huelsenbeck and Ronquist 2001), and posterior probability support for clades mapped on to the maximum likelihood trees. Depending on the value of the standard deviation of split frequencies, analyses were run for 200,000 to 1 million generations, with 25% discarded for burn-in. After separate analysis, sequences of 466 bp (βT-1), 322 bp (tef-1), 526 bp (rDNA-ITS), 449 bp (mt-LSU) and 513 bp (n-LSU) were concatenated and analysed by maximum likelihood, maximum parsimony and Bayesian methods. The maximum likelihood tree was constructed using DNAML, while the parsimony tree was constructed using DNAPARS. Bootstrap support for clades was obtained from 1000 replicates obtained using SEQBOOT and CONSENSE (PHYLIP package). Bayesian analysis was conducted as described above, with separate partitions for the nLSU, mtLSU, β-tubulin, tef-1, ITS1, 5.8S and ITS2 regions. The analysis was run for 1 million generations, with seven unlinked, unconstrained partitions, five heated chains, Nswaps=2, nst=6, rates=invgamma and Temp=0.1. All other parameters were set at default values. Summary statistics and a consensus tree were obtained after discarding the first 250,000 generations for burn-in. Trees were viewed with the TreeView software (Page

1996) and rooted using outgroup taxa. Trees and alignments were submitted to TreeBASE with study ID 15159 (http://treebase.org/treebase-web/search/study/summary.html?id=15159).

IV.3. RESULTS

IV.3.1. Phylogenetic analysis of individual genes

Levels of sequence variation among *Cyclaneusma* and *Lophodermium* species varied depending on the gene region, with translation elongation factor 1 (*tef-1*) sequences having the highest level of variation. *Tef-1* sequences from *C. minus* 'verum' and *C. niveum* differed by less than 1%, and *C. minus* 'simile' could be easily aligned with the other *Cyclaneusma* species. Similarly, the sequences from *Lophodermium*, *Ceuthospora* and *Strasseria* species were readily aligned with each other, but the alignment between the ingroup and outgroup is dubious due to the high variation (Figure 1).

Maximum likelihood phylogenetic trees of each gene region consistently separated isolates of *Cyclaneusma* spp. into three clades, corresponding to *C. minus* morphotype 'verum', *C. minus* morphotype 'simile' and *C. niveum* (Figure 1- Figure 5). The support for distinction of *C. minus* 'simile' from the other two species was strong (PP ranging from 0.97 to 1.00) but support for distinction of *C. niveum* and *C. minus* 'verum' was weak in the majority of the analyses (PP between 0.66 and 0.99).

In all five trees the *C. minus* morphotype 'verum' clade was closer to *C. niveum* than to *C. minus* morphotype 'simile'. Sequence variation between *C. minus* 'simile' and *C. minus* 'verum' ranged from 2 to 10%, depending on the gene region. An inserted fragment of 192 bp occurred near the end of the 18S region in all the *C. minus* 'simile' and *C. niveum* isolates but not in any of the *C. minus* 'verum' isolates. As introns in this region

may be transferred horizontally (Feau et al. 2007), this region was not included in the phylogenetic analysis. The relationships between *Cyclaneusma* species and candidate outgroup taxa were unclear. Nuclear gene regions placed the *Cyclaneusm*a clade closest to *Ceuthospora pinastri* (Helotiales) and *Strasseria geniculata* (incertae sedis), but the mitochondrial LSU placed it closer to the *Lophodermium species* (*Rhytismatales*).

IV.3.2. Multigene phylogenetic analysis

Visual inspection of single gene trees revealed the concordance of the three *Cyclaneusma* clades. Support for three distinct clades was low in some analyses (Figures 1, 3, 5) but there was no discordance. The multigene phylogeny determined by maximum parsimony, maximum likelihood and Bayesian analyses (Figure 6) replicated the groupings created by individual gene analyses and provided stronger support for the distinction of *C. minus* 'verum' and *C. niveum*. Phylogenetic analyses in this study supported two distinct groups of isolates encompassed within the fungal species currently treated as *C. minus.* Isolates from New Zealand and Australia fell into both clades. The three isolates from Germany and Kenya grouped with the *C. minus* 'verum' isolates.

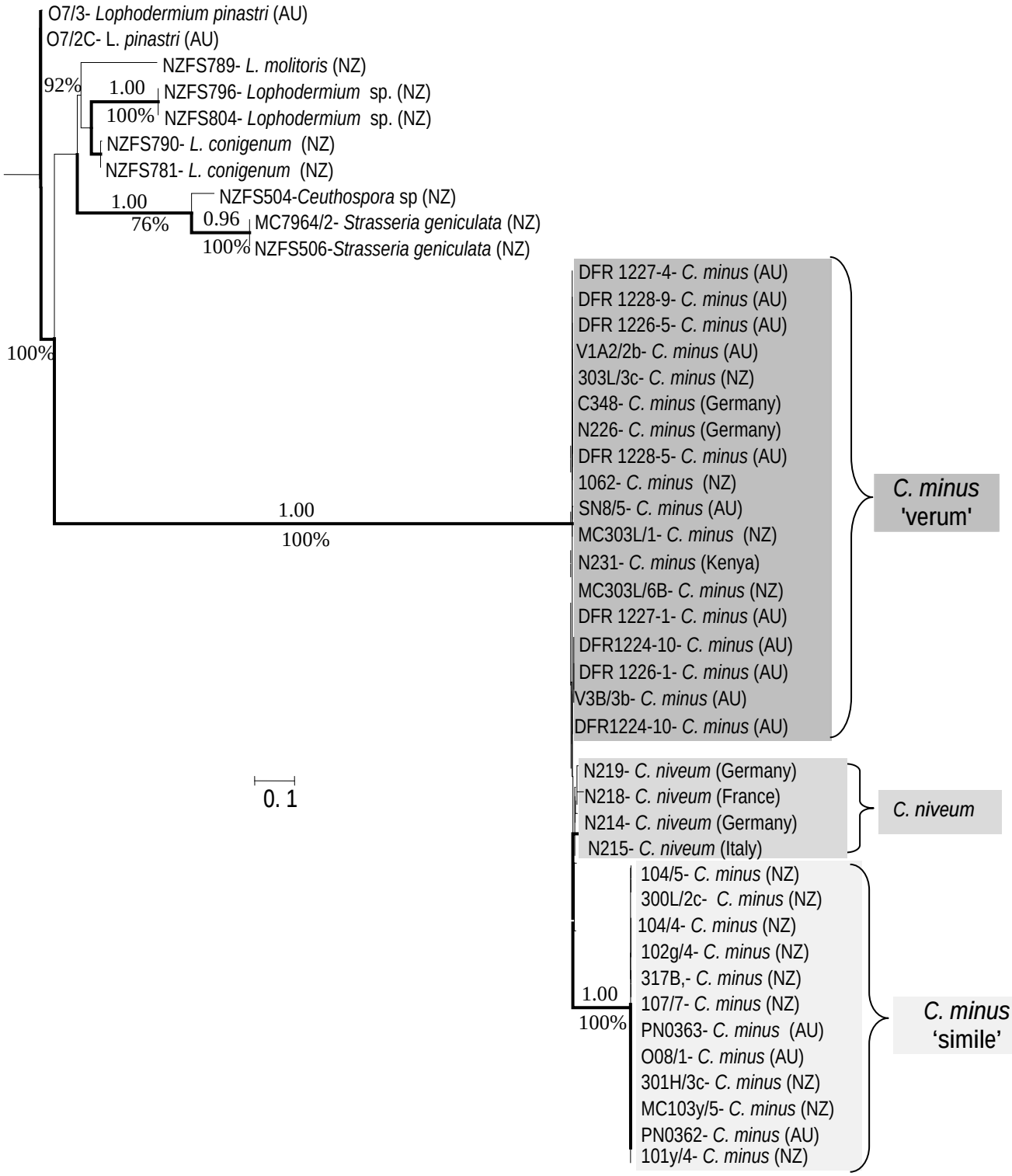

Figure 1. Maximum likelihood analysis of Translation Elongation Factor 1 (*tef-1*) gene sequences, Ln Likelihood = -2244.65657. Bar represents expected nucleotide variation of 10%. Bold branch lines indicate statistical support at P<0.01Bayesian posterior probabilities > 0.95 are shown above branches. Maximum parsimony analysis produced 5 most parsimonious trees of 324 steps, with the same overall topology as the ML tree, bootstrap support above 70% is indicated below branches. The *C. minus* 'verum' clade had 62% bootstrap support and *C. niveum* 50%. Abbreviations for country of origin are AU, Australia and NZ, New Zealand.

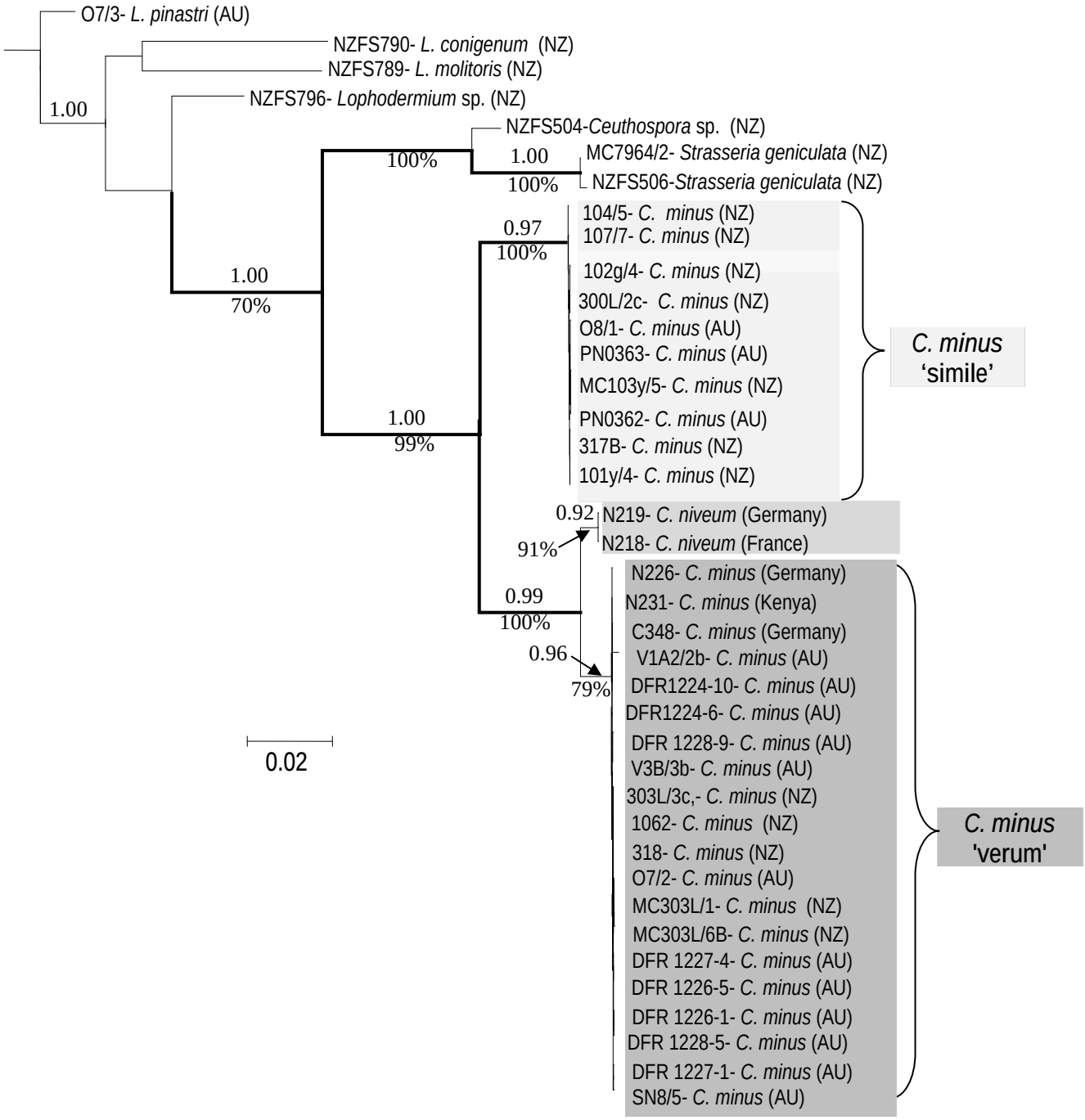

Figure 2. Maximum likelihood analysis of β-tubulin 1 (βT-1) gene sequences, Ln Likelihood = -2082.28641. Bar represents expected nucleotide variation of 2%. Bold branch lines indicate statistical support at P<0.01. Bayesian posterior probabilities > 0.90 are shown above branches. Maximum parsimony analysis produced five most parsimonious trees of 324 steps, with the same overall topology as the ML tree, bootstrap support above 70% is indicated below branches. Abbreviations for country of origin are AU, Australia and NZ, New Zealand.

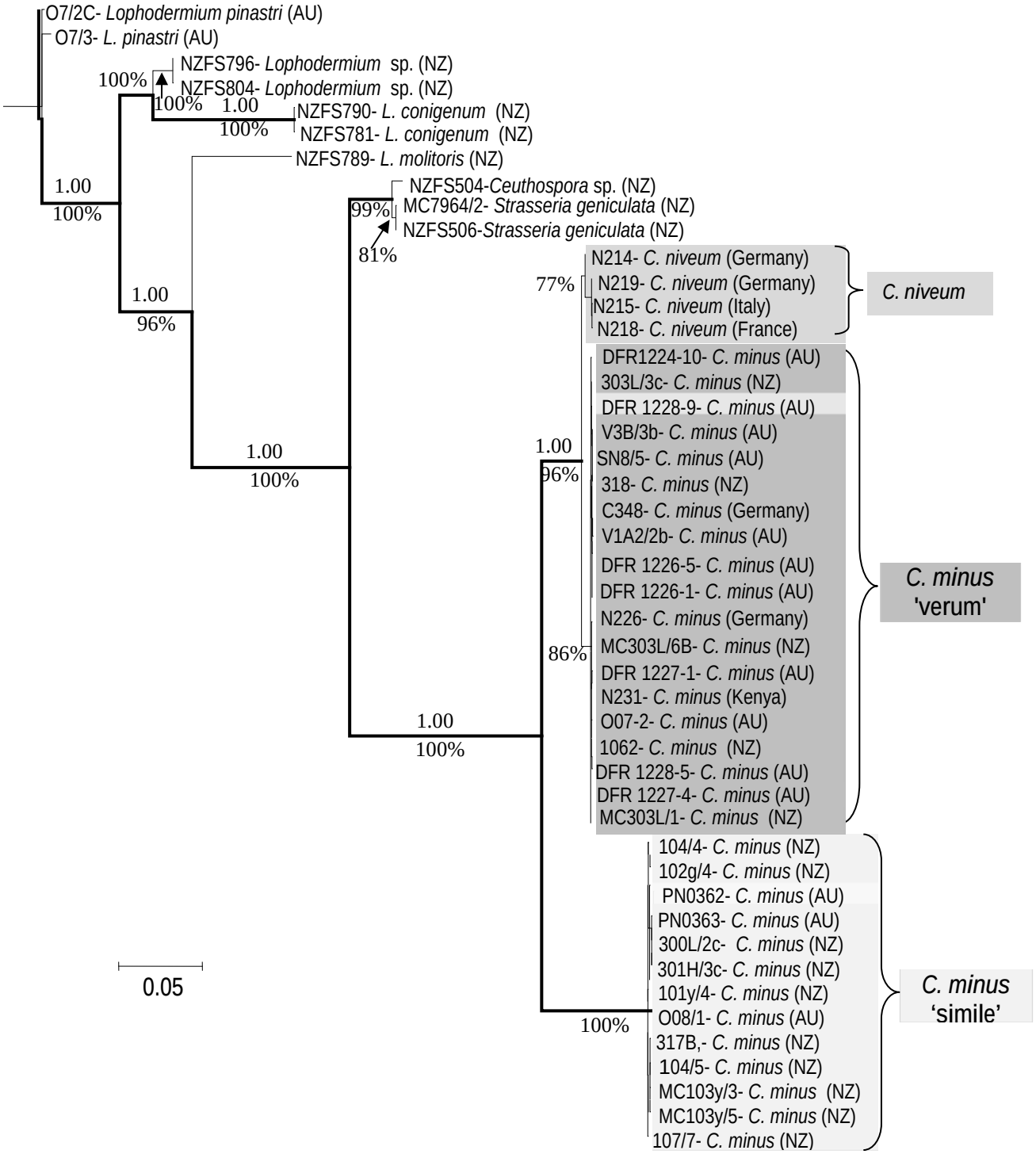

Figure 3. Maximum likelihood analysis of ribosomal DNA (rDNA) Internal Transcribed Spacer (ITS) sequences. Ln Likelihood = -2033.73971. Bar represents expected nucleotide variation of 5%. Bold branch lines indicate statistical support at P<0.01. Bayesian posterior probabilities > 0.95 are shown above branches. Maximum parsimony analysis produced one most parsimonious trees of 331 steps, with the same overall topology as the ML tree, bootstrap support above 70% is indicated below branches. Abbreviations for country of origin are AU, Australia and NZ, New Zealand.

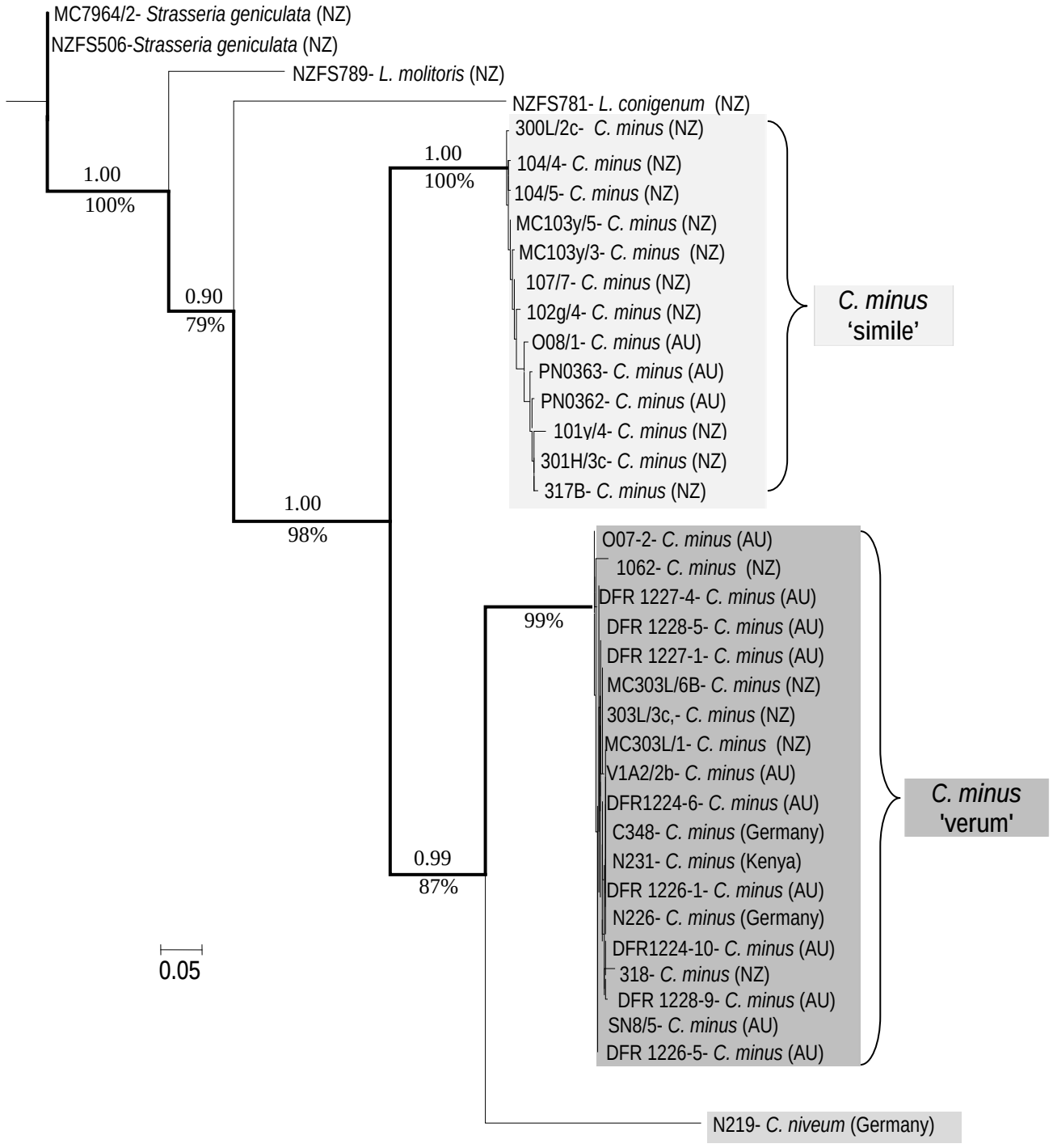

Figure 4. Maximum likelihood analysis of Mitochondrial Large Sub Unit (mt-LSU) rDNA sequences. Ln Likelihood = -2834.14275. Bar represents expected nucleotide variation of 5%. Bold branch lines indicate statistical support at P<0.01. Bayesian posterior probabilities > 0.95 are shown above branches. Maximum parsimony analysis produced ten most parsimonious trees of 564 steps, with the same overall topology as the ML tree, bootstrap support above 70% is indicated below branches. Abbreviations for country of origin are AU, Australia and NZ, New Zealand.

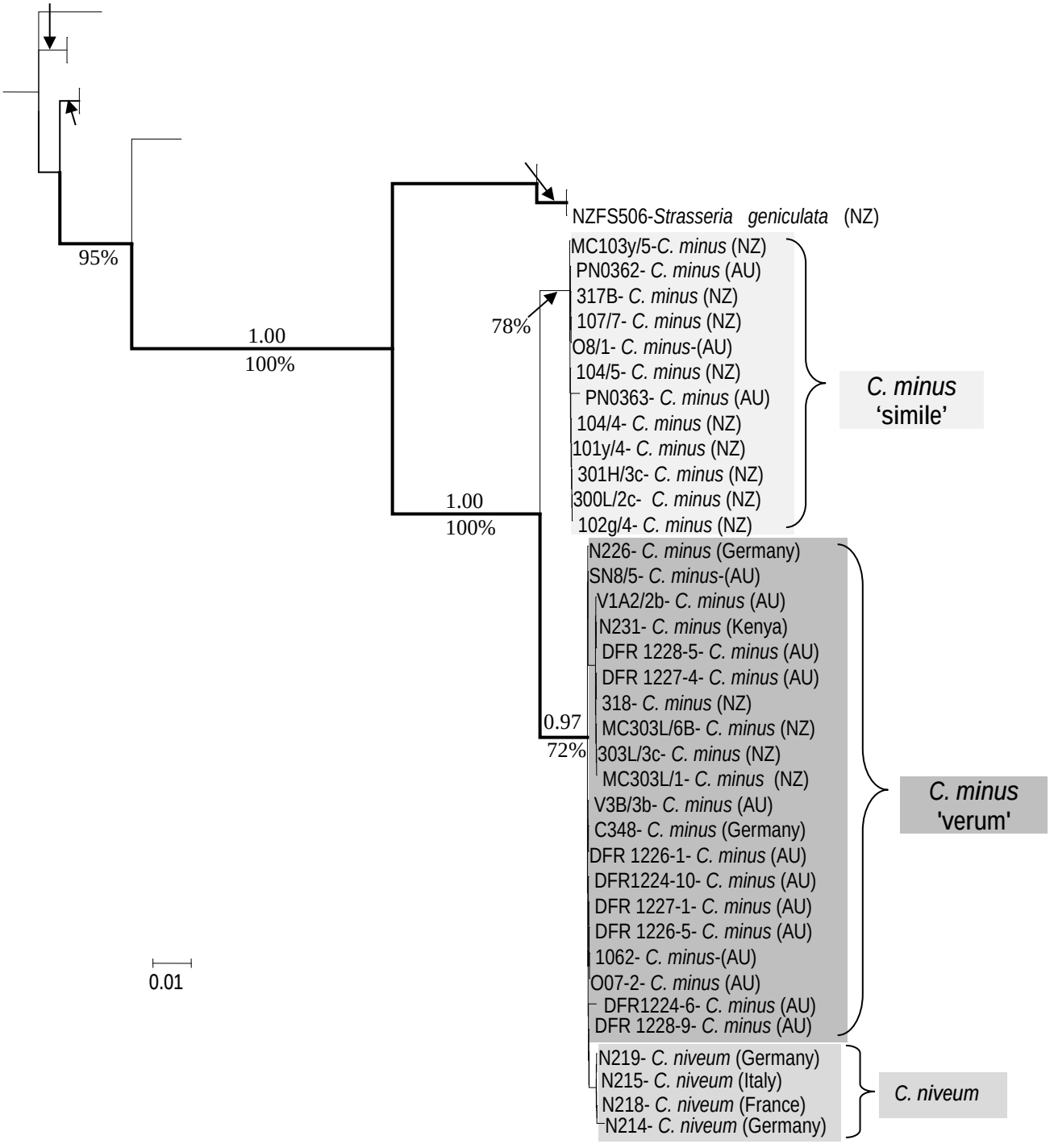

Figure 5. Maximum likelihood analysis of Nuclear Large Sub Unit (n-LSU) rDNA sequences. Ln Likelihood = -1369.25094. Bar represents expected nucleotide variation of 1%. Bold branch lines indicate statistical support at P<0.01. Bayesian posterior probabilities > 0.95 are shown above branches. Maximum parsimony analysis produced 14 most parsimonious trees of 131 steps, with the same overall topology as the ML tree, bootstrap support above 70%, based on 1000 resampled datasets, is also indicated below branches. Abbreviations for country of origin are AU, Australia and NZ, New Zealand.

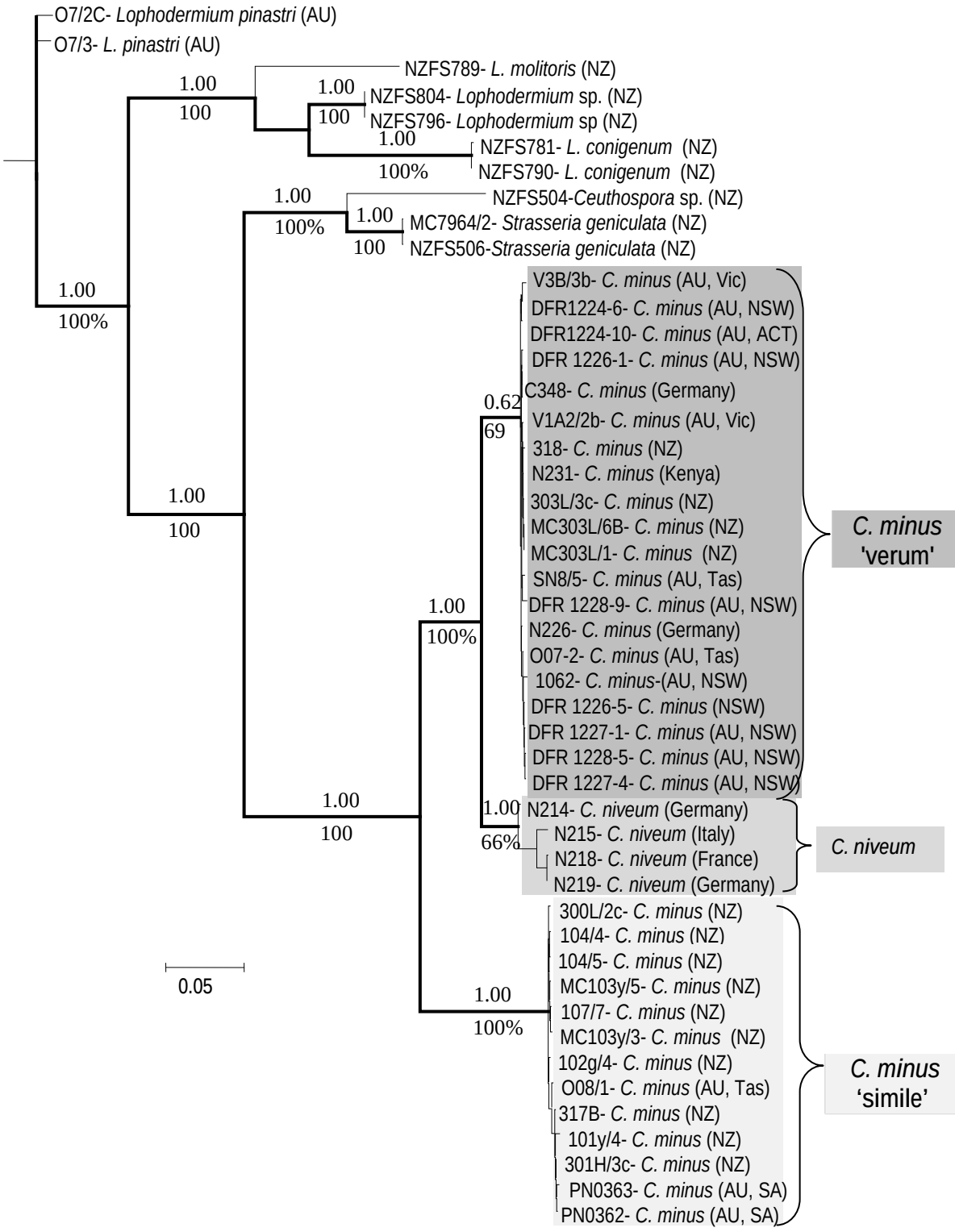

Figure 6. Maximum likelihood analysis based on an alignment of the combined data set from five genes (βT-1, *tef-1*, rDNA ITS, mt-LSU and n-LSU). Ln Likelihood = -12076.79393. Bar represents expected nucleotide variation of 5%. Bold branch lines indicate statistical support at P<0.01. Bayesian analysis produced a tree with the same topology (Ln Likelihood = -12800) and posterior probabilities are included below branches. Bootstrap figures from the maximum parsimony analysis, based on 1000 resampled datasets, are included above branches. The only difference in tree topology

between ML and MP analyses was the placement of *L. molitoris* in relation to other *Lophodermium* species. Abbreviations for country of origin are AU, Australia and NZ, New Zealand. For Australian isolates, the state of origin is also provided: ACT, Australian Capital Territory; NSW, New South Wales; SA, South Australia; Tas, Tasmania; Vic, Victoria.

IV.4. DISCUSSION

Despite the increase in genome-wide phylogenetic analyses (Rokas and Carroll 2005), most multigene phylogenetic analyses of fungi are based on up to five gene regions (Boehm et al. 2009; Crous et al. 2009; Taskın et al. 2010; Vialle et al. 2013). In this phylogenetic analysis, DNA sequences from five gene regions clearly showed that isolates currently classified as *C. minus* belong to two different species. The two clades of *C. minus* are consistent with the two different morphotypes of *C. minus* reported in a previous study (Bulman and Gadgil 2001). One morphotype ('verum') is more closely related to *C. niveum* than to the other morphotype ('simile') which should receive a formal description as a new species.

Distinction at the species level is also supported by (i) *C. minus* 'simile' is a sister taxon to a clade containing *C. minus* 'verum' and *C. niveum;* (ii) longer branch length separating *C. minus* 'simile' from the other two species; and (iii) higher bootstrap and posterior probability support for the distinction of *C. minus* 'simile' from the other two species than between *C. minus* 'verum' and *C. niveum.* As *C. minus* 'simile' constitutes a sister group to the clade containing *C. minus* 'verum' and *C. niveum*, the phylogenetic analysis cannot determine whether the new species belongs to *Cyclaneusma* or another genus. Determination of the appropriate genus will depend on taxonomic studies of herbarium specimens including ascocarps and ascospores, but as both species have been considered to represent *C. minus*, it is most likely that they belong to the same genus.

Distinction of *C. minus* 'verum' from *C. niveum* received a lower level of support than did the distinction of the two *C. minus* clades, but was supported by the βT1, *tef-1* and multigene analyses. Other studies (e.g. Vialle et al. 2013) have used genealogical concordance phylogenetic species recognition (GC-PSR) to support species discrimination but simulations have shown that a more accurate tree is obtained by analysing concatenated sequences, even when sequences have evolved in very different ways and no accommodation is made for the different evolutionary models (Gadagkar et al. 2005). We used a multigene analysis but genealogical concordance of the five gene regions is clear from visual inspection of the single gene trees.

Only three Tasmanian isolates of *C. minus* were included in this study but these separated into two different clades, confirming that the two species corresponding to the morphotypes *C. minus* 'verum' and 'simile' are present in plantations of *P. radiata* in Tasmania and in New Zealand. Although two distinct morphotypes of *C. minus* have been described for over a decade, little is known about their biology. The origin and geographical range of *C. minus* 'simile' are undetermined. *Cyclaneusma minus* 'verum' was the most common (Bulman and Gadgil 2001) type identified in New Zealand in a survey of *P. radiata* between 1977 and 1983. However in a recent survey in Tasmania of *P. radiata* needles sampled from trees with symptoms of Spring Needle Cast, the most frequently detected *Cyclaneusma* species was 'simile' (Prihatini, unpublished data). These results could lead to speculation that a difference in pathogenicity between the two species may be an important factor contributing to regional variation in severity of needle-cast. However, within some forests in New Zealand, sites with similar disease levels to those surveyed from 1977 to 1983 were sampled from 1996 to 1998 and proved to have quite different populations of 'verum' and 'simile'.

There are a number of possible reasons for variations in the predominance and pathogenic behaviour of similar species including host genotype, and/or environmental factors such as climate or the presence of fungal endophytes in needles which are reported in other systems to give protection against pathogens (Arnold et al. 2003; Ownley et al. 2010). Differences in survey methodology make previous studies difficult to compare i.e. bulk sampling of needles from trees or individual tree sampling masks host variation in disease susceptibility; previous reliance on morphological or cultural techniques could favour the identification and detection of one species over another. Previous studies into the correlation between disease levels, environmental factors and prevalence of the two morphotypes 'simile' and 'verum' in New Zealand were unable to draw conclusions because of the difficulty of accumulating sufficient data. It is now possible to design species-specific primers from the sequences available for the two 'verum' and 'simile' species and develop PCR-based diagnostic tests appropriate for large scale sampling direct from needle tissue, avoiding the laborious processes of isolation and morphological examination (Glen et al. 2007).

Cyclaneusma was considered to be a valid genus, distinct from *Naemacyclus* and the phylogenetic analyses did not include any *Naemacyclus* species. There are only two recognised species in *Cyclaneusma* whereas if *Cyclaneusma* is recombined into *Naemacyclus,* as proposed by Lumbsch and Huhndorf (2010) *Naemacyclus* would encompass 25 species excluding those that have recently been moved into other genera such as *Marthamyces* (Johnston 2006). Many of those 25 species are known only from their type specimens, but the type species of the genus, *Naemacyclus fimbriatus*, is well known as a saprophyte of the cones of several pine species and has been recorded from needles of *Pinus rigida* (Vujanovic et al. 1998). A phylogenetic analysis based on 18S rDNA sequences grouped *C. minus* and *N. fimbriatus* in a well-supported clade with

approx. 2% sequence divergence across a 1 kbp fragment of small subunit nrDNA (Gernandt et al. 2001), but the sequence for *N. fimbriatus* used in that study (AF203457.1) has less than 90% sequence similarity to another accession (FJ176811.1), purportedly from the same isolate (CBS 289.61) (Schoch et al. 2009). It is unclear which accession represents the correct sequence for that isolate.

The 'outgroup', usually a 'sister' species, is difficult to determine for *Cyclaneusma*. Previous studies (Lantz et al. 2011; Schoch et al. 2009) indicate that *Naemacyclus fimbriatus (Helotiales)* would be a suitable outgroup for *Cyclaneusma,* but appropriate sequence data, DNA or isolates of this species were unavailable. Until recently, *Cyclaneusma* was considered to belong to *Rhytismataceae* in the *Rhytismatales,* but is now considered a member of *Helotiales* incertae sedis (Schoch et al. 2009). Phylogenetic analysis of the nLSU and mtSSU of a small number of Helotiales and Rhytismatales species placed *Cyclaneusma* in a clade with species from both of these orders (Lantz et al. 2011). We included *Ceuthospora* sp. (*Helotiales*), *Strasseria geniculata* (incertae sedis) and *Lophodermium* spp. (*Rhytismatale*s) as outgroups and most analyses placed *Cyclaneusma* closer to *Ceuthospora* and *Strasseria* than to *Lophodermium,* but the relationships among these taxa was not the main focus of the study.

Confirmation of the occurrence of two *Cyclaneusma* species on *P. radiata* in Australia and New Zealand and their molecular characterisation will facilitate examination of the role each species plays in diseases such as Cyclaneusma Needle Cast (Watt et al. 2012) and Spring Needle Cast (Podger; Wardlaw 1990) and their interactions with environmental conditions. Both New Zealand and Australia face increasing climate

variability and changes to seasonal patterns of rainfall (e.g. Holz et al 2010) which could influence host-pathogen interactions and disease severity.

V. SURVEY OF NEEDLE FUNGI IN *PINUS RADIATA* TREES WITH VARYING LEVELS OF RESISTANCE TO SPRING NEEDLE CAST

Abstract

Spring needle cast (SNC) in Tasmania is defined by the early casting of pine needles and appears in 6-7 years-old pine plantations after canopy closure. The severity of SNC can vary significantly among trees growing within the same plantation. Fungal communities were surveyed in a *Pinus radiata* family trial, using direct PCR and sequencing to detect the presence of fungal species. Samples were taken at the same time as the trees were scored for disease severity. Trees with contrasting levels of SNC disease severity have significantly different needle fungal communities, but family pedigree and different ages of needle are not clearly distinguished by their fungal communities. All common fungal pathogens previously implicated in causing SNC were identified from the study, but of these, only *Lophodermium pinastri* was correlated with high levels of disease.

V.1. INTRODUCTION

Spring needle cast (SNC) in Tasmania is defined by the early casting of pine needles in plantation *Pinus radiata* D. Don which follows the development of yellow and brown mottling. The symptoms start in early spring and appear in young pine plantations after canopy closure (age 6-7 years), especially in wet areas at high altitude (Podger and Wardlaw 1990a). Spring needle cast leads to significant growth reductions (Podger and Wardlaw 1990b), especially in affected clearwood (pruned and thinned) plantations during the latter half of their rotation (Kube and Wardlaw 2002a).

The causal agent(s) of SNC in Tasmanian have not been clearly identified (Podger and Wardlaw 1990a). No specific fungal fruit bodies are consistently present in the needles of trees with SNC, but sometimes the fruit bodies of *Lophodermium pinastri* and

Cyclaneusma minus are observed (Podger and Wardlaw 1990a). Spring needle cast is not considered to be a classical needle blight disease caused by a primary fungal pathogen. It is thought to be caused by a suite of endophytic fungi that are triggered into secondary pathogenic activity by an environmental stress (Podger and Wardlaw 1990a). Three fungal species are considered to play a role in SNC in Tasmania i.e. *C. minus***,** *Strasseria geniculata* and *L. pinastri* (Podger and Wardlaw 1990a). A recent phylogenetic analysis (Prihatini et al. 2014a) supports the existence of two separate species in *C. minus,* previously recognised as distinct cultural morphotypes, termed *C. minus* 'verum' and *C. minus* 'simile' (Dick 2001). Many *Lophodermium* species (Ganley 2004a) and other Rhytismataceae species may remain dormant until climatic conditions become more conducive to disease outbreaks, or may switch to pathogenic behaviour in stressed trees (Moricca and Ragazzi 2007). Conversely, certain endophytic needle fungi may play a role in limiting the damage caused by pathogens and this type of role is becoming of considerable interest and the subject of recent research in forestry (Santamaria et al. 2007, Ganley et al. 2008, Eyles et al. 2010), particularly in *P. radiata* (Reay et al. 2010, Burdon 2011, Brownbridge et al. 2012).

Resistance to SNC varies among *P. radiata* populations. There is clear evidence that resistance to this disease is inherited, thus breeding selection programs may offer the best solution to reduce the impact of SNC in the long term (Podger and Wardlaw 1990a). Several studies have explored forest-tree defence mechanisms and the deployment of resistance, though these are mainly limited to a few commercially important timber species like *Populus, Pinus, Picea, Eucalyptus,* and *Pseudotsuga* (Witzell and Martín 2008, Duplessis et al. 2009). Silvicultural selection for desirable wood traits may not coincide with selection for resistance, in part because of a reduction of chemical and genetic diversity (Carraway 2001). Selection of trees for resistance to insect pests and pathogens

requires an assessment of trial plantations for heritable variation in levels of damage. Identifying rare resistant genotypes using markers may be difficult because many factors interact to produce a resistant phenotype (Henery 2011). The severity of Spring Needle Cast (SNC) can vary significantly among trees in the same plantation (Podger and Wardlaw 1990a). Assessments of resistance to SNC show that resistance is moderately heritable, with values of 0.253 and 0.37 measured in Tasmanian studies (Podger and Wardlaw 1990a, Kube and Wardlaw 2002b).

The breeding values calculated from SNC assessments made in one of these studies (Kube and Wardlaw 2002b) were used to select parental crosses for a Marker Aided Selection trial. Marker aided selection (MAS) is a process whereby a marker (morphological, biochemical or one based on DNA/RNA variation) is used for the indirect selection of a genetic determinant or determinants of a trait of interest (i.e. productivity, disease resistance, abiotic stress tolerance, and/or quality).The MAS trial was established in 1999 at Oonah, Tasmania, to allow future studies into the genetic mechanisms of *P. radiata* resistance to SNC (Kube and Piesse 1999). Trees within one replicate of this trial were evaluated for disease severity in 2007, when SNC was in the early stages of expression (Wardlaw et al. 2007).

The objective of this study was to characterise the fungal communities associated with needles on trees of *P. radiata* scored for SNC damage in the MAS trial and to determine whether particular fungal species or communities were associated with high or low disease levels and/or the host pedigree. Needle-inhabiting fungi were characterised with the aid of molecular tools to assist in establishing which species may be causal agents of SNC.

V.2. MATERIALS AND METHODS

V.2.1. Needle samples

Pine needle samples were collected in spring 2007 from the *P. radiata* SNC Marker Aided Selection (MAS) trial that was planted in June 1999 by Forestry Tasmania in Oonah, North West Tasmania. This site has a mean annual rainfall of 1655 mm; mean daily temperature 9.9 °C; altitude > 350 m (Kube and Piesse 1999). The trial includes three full-sib families with known breeding values for SNC resistance (Table 1). The trees were scored for SNC severity immediately before sample collection. Trees were assigned to 1 of 4 different disease classes depending on the severity of needle-cast: 1= < 5% needle-cast; 2 = 5-<30% needle-cast; 3 = 30-<60% needle-cast;4 = >60% needle-cast (Wardlaw et al. 2007). Within each family, a random selection of three trees of each of four disease classes (1-4) were sampled, i.e. 12 trees per family, giving a total of 36 trees. From each tree three different ages of needle were collected from the lower crown, providing a total of 108 bulk needle samples.

Table 1.The three full-sib families[1] used in the Oonah MAS trial (Kube and Piesse 1999).

Family number	Family pedigree	Number of progeny (seedlings)	Number of trees from cuttings	Source
1	12038 x 70053	490	1954	Forestry Tasmania
2	20002 x 20055	518	2057	Forestry Tasmania
3	31053 x 31032	368	1424	CSIRO

[1]Families 1 and 2 each had one parent with high and one parent with low susceptibility to SNC (Kube and Piesse 1999). Family 3 was the CSIRO mapping pedigree (Devey et al. 1996).

V.2.2. DNA extraction

From each bulk sample, five needles were randomly selected from different fascicles. Approximately 1 cm was cut from the middle section of each needle and placed into a 1.5 mL microcentrifuge tube and stored at -80°C prior to DNA extraction. Needles were ground using a porcelain mortar and pestle with liquid nitrogen added to facilitate grinding. DNA extraction was performed using SDS extraction buffer (Raeder and Broda 1985) and purified using glassmilk (Boyle and Lew 1995) according to Glen et al. (2002).

V.2.3. Polymerase Chain Reaction (PCR) and electrophoresis

Fungal DNA was amplified using primers targeting the rDNA ITS (ribosomal DNA internal transcribed spacers). The ascomycete-specific primer ITS4A (Larena et al. 1999) was paired with ITS5 (White et al. 1990). PCR was conducted in 50 μL reactions with a final concentration of ingredients as follows: 67mM Tris-HCl, pH 8.8; 16mM $(NH_4)_2SO_4$ (in 10× NH_4-based reaction buffer supplied by Bioline, UK); 2.0 mM magnesium chloride (Promega, USA); 200 μM deoxynucleotide triphosphate (Bioline, UK); 0.25 μM oligonucleotide primer (Geneworks, Australia); 0.02 units/μL of Mangotaq DNA polymerase (Bioline, UK); 0.2 μg/μL of bovine serum albumin (Fisher Biotech, Australia) to reduce enzyme inhibition that may be present in the DNA template (Kreader 1996); 10 μL of the diluted DNA in TE (1/40, 1/60 and 1/100) and sterile water (Astra Zeneca, Australia) to make up to a volume of 50 μL. Amplification was performed using a Peltier Thermal Cycler PTC-225 (MJ Research) and the following temperature profile: 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 2 min, with a final 7 min extension at 72°C. Nested PCR was performed on samples which had insufficient product for cloning after a single round of PCR. The product of PCR using ITS4A and

ITS5 was diluted 1 in 5 in TE buffer and used as a template in the second-round PCR with primers ITS1 and ITS4 (White et al. 1990). The reaction conditions and reagent concentrations for nested PCR were as described for the first-round PCR except the number of cycles was reduced to 20.

PCR products were separated by electrophoresis on a 1% agarose gel (Fisher Biotech, Australia) at 10 volts/cm for 30 min in a MI-DEAR 120 High Performance Gel System (Biokeystone, USA). Digested PCR products were separated for 2 h at 30 V/cm in a 3% high resolution agarose gel (Fisher Biotech, Australia). Gels were visualized on a Vilber Lourmat transilluminator after staining with ethidium bromide 0.5 μg/mL (MOBIO, USA) for 20 min and images captured using a Vilber Lourmat camera (Cedex, France).

V.2.4. Pooling and cloning of amplified DNA

To reduce the number of cloning reactions required, and to target the dominant fungal species, PCR products from needles of the same age from trees of the same disease class and the same family were pooled (Table 2). Cloning of PCR products was performed before sequencing to separate the templates of the several different fungi that may occur within the sample that included sections of fifteen pine needles from three trees. Pooled amplification products were cloned using a commercial kit, p-GEM®-T Easy Vector (Promega, USA) as described in the manufacturer's protocol.

Table 2. Pooling of PCR product from pine needle samples collected from the Oonah SNC MAS trial.

Family	Disease Severity Score	Needle Age	Tree Nos.	Needle Samples[1]	DNA Pool
10238 x 70053	1	1	155; 219; 445	ON01; ON04; ON07	1
10238 x 70053	1	2	155; 219; 445	ON02; ON05; ON08	2
10238 x 70053	1	3	155; 219; 445	ON03; ON06; ON09	3
10238 x 70053	2	1	127; 285; 416	ON28; ON31; ON34	10
10238 x 70053	2	2	127; 285; 416	ON29; ON32; ON35	11
10238 x 70053	2	3	127; 285; 416	ON30; ON33; ON36	12
10238 x 70053	3	1	221; 304; 503	ON49; ON52; ON55	19
10238 x 70053	3	2	221; 304; 503	ON56; ON59; ON62	20
10238 x 70053	3	3	221; 304; 503	ON57; ON60; ON63	21
10238 x 70053	4	1	99; 181; 248	ON82; ON85; ON88	28
10238 x 70053	4	2	99; 181; 248	ON82; ON85; ON88	29
10238 x 70053	4	3	99; 181; 248	ON82; ON85; ON88	30
20002 x 200055	1	1	1167; 1343; 1458	ON10; ON13; ON16	4
20002 x 200055	1	2	1167; 1343; 1458	ON11; ON14; ON17	5
20002 x 200055	1	3	1167; 1343; 1458	ON12; ON15; ON18	6
20002 x 200055	2	1	1265; 1434; 1536	ON37; ON40; ON43	13
20002 x 200055	2	2	1265; 1434; 1536	ON38; ON41; ON44	14
20002 x 200055	2	3	1265; 1434; 1536	ON39; ON42; ON45	15
20002 x 200055	3	1	1010; 1225; 1259	ON64; ON67; ON70	22
20002 x 200055	3	2	1010; 1225; 1259	ON65; ON68; ON71	23
20002 x 200055	3	3	1010; 1225; 1259	ON66; ON69; ON72	24
20002 x 200055	4	1	1216; 1221; 1359	ON91; ON94; ON97	31
20002 x 200055	4	2	1216; 1221; 1359	ON92; ON95; ON98	32
20002 x 200055	4	3	1216; 1221; 1359	ON93; ON96; ON99	33
31053x31032	1	1	14733; 14764; 14768	ON19; ON22; ON25	7
31053x31032	1	2	14733; 14764; 14768	ON20; ON23; ON26	8
31053x31032	1	3	14733; 14764; 14768	ON21; ON24; ON27	9
31053x31032	2	1	14634; 14690; 15083	ON46; ON49; ON52	16
31053x31032	2	2	14634; 14690; 15083	ON47; ON50; ON53	17
31053x31032	2	3	14634; 14690; 15083	ON48; ON51; ON54	18
31053x31032	3	1	14457; 14677; 14773	ON73; ON76; ON79	25
31053x31032	3	2	14457; 14677; 14773	ON74; ON77; ON80	26
31053x31032	3	3	14457; 14677; 14773	ON75; ON78; ON81	27
31053x31032	4	1	14450; 14775; 14834	ON100; ON103; ON106	34
31053x31032	4	2	14450; 14775; 14834	ON101; ON104; ON107	35
31053x31032	4	3	14450; 14775; 14834	ON102; ON105; ON108	36

[1]Each needle sample code represents duplicate samples consisting of one needle from each of five fascicles of the same age.

Transformed bacterial colonies containing plasmid inserts were screened by PCR-RFLP to select samples for sequencing. Thirty-two colonies from each cloning reaction were randomly selected for subculture onto new 1% LB agar with Ampicillin (Sigma-

Aldrich, Sydney, NSW), IPTG (Promega, USA) and X-gal (Sigma-Aldrich, Australia) and then incubated at 37°C overnight.

A rapid DNA extract was prepared by dipping a sterile toothpick in a colony, then twirling it in 50 µl of TE buffer in one well of a 96-well plate. An aliquot was then used for PCR amplification of inserts. Five µl of PCR products were digested using restriction enzymes Alu I (Promega, USA) and Hin FI (Promega, USA) in a final volume of 10 µl then electrophoresed on a 3% high resolution agarose gel (Promega, USA) at 10 volts/cm for 5 hours. Products of clones from the same transformation reaction were electrophoresed on the same gel to facilitate visual assessments of groups according to their RFLP patterns and two or three clones from each PCR-RFLP group were randomly selected for sequencing.

V.2.5. DNA sequencing and phylogenetic analysis

DNA sequencing of PCR products was outsourced to Macrogen Inc (http://dna.macrogen.com/eng/). Chromatograms were viewed in ChromasPro version 1.34 software and edited to remove poor quality sequences at each end and then the sequence was saved in FASTA format. A BLAST (Basic Local Alignment Search Tool) search of public DNA databases retrieved sequences of high similarity (Altschul et al. 1990).

Isolates or clones with less than 2% sequence variation were grouped into operational taxonomic units (OTUs). The fungi or OTUs were identified to the lowest possible taxonomic level based on sequence similarity to known fungal isolates or sequences from public DNA databases. Phylogenetic analysis helped to refine the discrimination and identification of OTUs (Prihatini et al. 2014b). Sequences of high similarity were aligned using ClustalW (Thompson et al. 1994). If single nucleotide

polymorphisms occurred between colonies or clones, chromatograms were rechecked to confirm these. OTUs with less than 99% similarity to a robustly identified isolate were identified only to genus or family level, or higher (Table 3).

V.2.6. Statistical analysis

Each OTU observed in this study, including singletons, was scored as present or absent in each pooled sample representing an age-class of needles from three trees with the same disease severity rating in each of the three families. Prevalence of OTUs was tallied for each disease class, each family and also each needle age group. The presence and absence data were also used in a statistical analysis using primer v6 with PERMANOVA+ software (Anderson et al. 2008). PERMANOVA analysis was conducted to determine any significant differences in fungal communities in needles of different ages and from trees in different disease classes or from different families. PERMANOVA is an analysis of variance using permutations (as opposed to f-tests in the traditional ANOVA approach where one has data that can be considered to be 'normally distributed' (Anderson et al. 2008). The pairwise tests between each pair of components were also conducted if PERMANOVA analysis indicated there was a significant correlation. A canonical analysis of principal co-ordinates (CAP) was also conducted as the graphical output facilitates visualisation of the results.

V.3. RESULTS

V.3.1. Fungal OTUs and their prevalence

A total of 65 fungal OTUs were discriminated, with 36 occurring in more than one pooled sample (Table 3). The prevalence of each OTU in samples from different host families, needle ages and disease class was used as the basis for statistical analyses. The prevalence of 16 dominant OTUs, which were present in more than four out of 36 pooled samples are presented in Table 4. Teratosphaeriaceae sp. 23 was the most commonly detected fungus and was present in all four disease classes, all three needle ages and all three families. It appears slightly more prevalent in healthier trees and younger needles although there is no strong affinity to any family, needle age or disease severity class. *C. minus* 'simile' was the second most common fungus and was present in approximately half of the samples equally across needle ages and disease classes but was more prevalent in family 3.

Table 3. List of all OTUs observed in this study with the number of pooled needle samples (from a total of 36) in which each OTU was detected. Each pooled sample represents 15 needle samples from 3 trees, all of the same needle age, tree family and disease severity scores.

Class; Order	Family	OTU	No. of samples	GenBank Accession
Ascomycetes; *incertae sedis*	*incertae sedis*	Ascomycete sp. 1	8	KM216362
		Ascomycete sp. 2	11	KM216358
		Ascomycete sp. 4	1	KM216378
		Ascomycete sp. 5	1	KM216363
		Ascomycete sp. 6	1	KM216360
		Strasseria geniculata (Berk. & Broome) Höhn.	6	KJ406932
Dothideomycetes; Botryosphaeriales	*incertae sedis*	Botryosphaeriales sp. 1	2	KM216369
Dothideomycetes; Capnodiales	Davidiellaceae	*Davidiella* sp.	3	KM216381
	incertae sedis	Capnodiales sp. 1	1	KJ406762
		Capnodiales sp. 2	1	KM216384
		Phaeotheca fissurella Sigler, Tsuneda & J.W. Carmich.	13	KM216356
		Dothistroma septosporum (Dorog.) M. Morelet	16	KJ406808
	Mycosphaerellaceae	Mycosphaerellaceae sp. 1	2	KJ406789
		Mycosphaerellaceae sp. 2	2	KJ406794
		Phaeothecoidea sp. 3	2	KJ406796
	Teratosphaeriaceae	*Catenulostroma* sp. 1	2	KM216372
		Devriesia sp.	1	KM216374
		Teratosphaeria parva (R.F. Park & Keane) Crous & U. Braun	4	KJ406775
		Teratosphaeriaceae sp. 1	4	KJ406774
		Teratosphaeriaceae sp. 2	2	KJ406778
		Teratosphaeriaceae sp. 3	12	KM216368
		Teratosphaeriaceae sp. 4	7	KJ406777

Class; Order	Family	OTU	No. of samples	GenBank Accession
		Teratosphaeriaceae sp. 8	2	KM216380
		Teratosphaeriaceae sp. 11	1	KJ406783
		Teratosphaeriaceae sp. 12	1	KJ406782
		Teratosphaeriaceae sp. 13	6	KJ406779
		Teratosphaeriaceae sp. 15	4	KJ406776
		Teratosphaeriaceae sp. 16	5	KM216367
		Teratosphaeriaceae sp. 18	4	KM216357
		Teratosphaeriaceae sp. 20	1	KJ406780
		Teratosphaeriaceae sp. 21	6	KM216361
		Teratosphaeriaceae sp. 22	3	KJ406781
		Teratosphaeriaceae sp. 23	33	KJ406772
		Teratosphaeriaceae sp. 24	5	KM216373
		Aureobasidium pullulans (De Bary) G. Arnaud ex Cif. Ribaldi & Corte	2	KJ406823
		Sydowia polyspora (Bref. & Tavel) E. Müll.	2	KJ406830
Dothideomycetes; Dothidiales	Dothioraceae	*Phoma* sp.	6	KJ406858
		Pleosporales sp.	1	KJ406833
Dothideomycetes; Pleosporales	*incertae sedis*	Didymellaceae sp.1	1	KJ406840
		Didymellaceae sp.2	2	KM216364
	Didymellaceae	*Lophiostoma corticola* (Fuckel) E.C.Y. Liew, Aptroot & K.D. Hyde	2	KJ406843
		Paraconiothyrium sp. 1	1	KJ406852
	Lophiostomataceae	*Paraconiothyrium* sp. 2	2	KJ406853
	Montagnulaceae	*Phaeosphaeria* sp. 1	1	KM216359
		Phaeosphaeria sp. 2	1	KM216376
	Phaeosphaeriaceae	*Alternaria eureka* E.G. Simmons	1	KM216382
		Sporormiella sp. 1	1	KJ406868

Class; Order	Family	OTU	No. of samples	GenBank Accession
	Pleosporaceae	*Sporormiella* sp. 4	1	KJ406866
	Sporormiaceae	*Sporormiella* sp. 5	1	tba
		Sporormiella sp. 6	1	KJ406864
		Exophiala sp. 1	1	KJ406883
		Exophiala sp. 2	1	KJ406882
Eurotiomycetes; Chaetothyriales	Herpotrichiellaceae	Chaetothyriales sp. 2	1	KJ406875
		Aspergillus conicus Blochwitz	1	KJ406886
	incertae sedis	*Aspergillus ustus* (Bainier) Thom & Church	6	KJ406896
Eurotiomycetes; Eurotiales	Trichocomaceae	Helotiales sp. 3	1	KM216383
		Helotiales sp. 5	1	KM216379
Leotiomycetes; Helotiales	*incertae sedis*	*Leptodontidium* sp.	1	KM216366
	Phacidiaceae	*Ceuthospora pinastri* (Fr.) Höhn.	1	KJ406933
Leotiomycetes; *incertae sedis*	*incertae sedis*	*Cyclaneusma minus* 'simile'	17	KJ406913
		Cyclaneusma minus 'verum'	2	KJ406908
Leotiomycetes; Rhytismatales	Rhytismataceae	*Lophodermium pinastri* (Schrad.) Chevall.	12	KJ406952
		Rhytismatales sp. 2	4	KJ406971
		Rhytismatales sp. 3	1	KJ406974
Saccharomycetes; Saccharomycetales	*incertae sedis*	*Candida sake* (Saito & Oda) van Uden & H.R. Buckley	1	KM216377

No specific fungi were consistently associated with young needles. Ascomycete sp. 2 and *Phaeotheca fissurella* were fungi detected more frequently in healthy than in diseased trees. *Lophodermium pinastri,* Teratosphaeriaceae sp. 3 and Teratosphaeriaceae sp. 13 were more commonly associated with trees in the higher disease classes.

Table 4. Prevalence of major fungal OTUs detectedfrom a total of 36 pooled samplesand tallied by: disease class, needle age and family pedigree. Species shown in bold have been historically considered as the causal agents of SNC disease (Wardlaw 2008).

OTUs	Disease severity score[1]				Needle age[2]			Family[3]		
	1	2	3	4	1	2	3	1	2	3
Teratosphaeriaceae sp. 23	9	9	7	8	12	11	10	10	12	11
***C. minus* 'simile'**	**4**	**4**	**4**	**5**	**6**	**6**	**5**	**4**	**4**	**9**
Dothistroma septosporum	4	6	2	4	6	3	7	6	4	6
Phaeotheca fissurella	5	4	2	2	4	3	6	6	3	4
Teratosphaeriaceae sp. 3	0	2	3	7	3	3	6	4	3	5
Lophodermium pinastri	**1**	**2**	**5**	**4**	**3**	**3**	**6**	**1**	**6**	**5**
Ascomycete sp. 2	5	3	1	2	3	4	4	3	5	3
Ascomycete sp. 1	0	4	2	2	4	2	2	3	2	3
Teratosphaeriaceae sp. 4	3	2	2	0	2	3	2	2	3	2
Strasseria geniculata	**2**	**1**	**0**	**3**	**2**	**0**	**4**	**3**	**2**	**1**
Aspergillus ustus	0	0	1	5	2	2	2	1	2	3
Teratosphaeriaceae sp. 21	1	2	1	2	2	1	3	2	3	1
Pleomassariaceae sp.	3	3	0	0	2	2	2	2	2	2
Teratosphaeriaceae sp. 13	0	1	1	4	1	3	2	1	2	3
Teratosphaeriaceae sp. 24	0	0	2	3	2	2	1	3	0	2
Teratosphaeriaceae sp. 16	0	3	1	1	2	2	1	1	2	2
***C. minus* 'verum'**	**0**	**0**	**1**	**1**	**0**	**2**	**0**	**0**	**1**	**1**

Note:

[1] The number of samples out of 9 pooled samples fromeach disease classin which an OTU was detected. Class1 = healthy trees, class2 = 5-30% defoliation, class3 = 30-60% defoliation and class4 = trees with >60% defoliation.

[2] The number of samples out of 12 pooled samples in each needle age (in years) in which an OTU was detected.

[3] The number of samples out of 12 pooled samples in each family group in which an OTU was detected. Family 1 (12038 x 70053), family 2 (20002 x 20055), family 3 (31053 x 31032)

Certain fungi, which were more prevalent in trees with higher disease severity, i.e. Teratosphaeriaceae sp. 3 and *L. pinastri*, were also found more frequently in older needles. *Dothistroma septosporum* was more frequently detected in healthy trees. *C. minus* 'simile' appeared more prevalent in family 3 (31053 x 31032) than the other two families. *Dothistroma* was more prevalent in family 1 (12038 x 70053) and family 3, while *L. pinastri* was more frequently detected in families 2 and 3. Family 1 had a higher incidence of *Phaeotheca fissurell*a. The incidence of other fungi associated with a particular family was too low and variable to show any particular trend. However a SNC survey conducted prior to this study found no significant correlation between SNC severity and tree family (Wardlaw, unpublished).

For those fungi previously implicated in SNC, only *L. pinastri* showed a greater prevalence in higher disease classes. *C. minus* 'simile' was more prevalent in family 3. *Strasseria geniculata* and *C. minus* 'verum' were only present in a small number of samples.

V.3.2. Fungal communities associated with needle age, family or disease class

The PERMANOVA analysis (Table 5) shows that significant differences in fungal communities were observed between different disease classes, but not between other single factors (needle age and tree family) or between the combinations of factors studied. Pair-wise tests for the disease class factor (data not shown) were used to examine the correlation of the fungal community between each pair of disease class. Fungal communities in trees from disease class1 (healthy) were significantly different (P=0.0108) from those in disease class4 (severely diseased).

Table 5.Permanova analysis of fungal communities observed in *Pinus radiata* Marker Aided Selection Trial at Oonah Tasmania

Source[1]	Degrees of freedom	Mean squares	Pseudo-F	P (perm)
di	3	5770.5	1.8457	0.0018
fa	2	3066.9	0.98095	0.4988
ne	2	3250.5	1.0397	0.4152
dixfa	6	2580.4	0.82533	0.8708
dixne	6	2760.9	0.88307	0.7625
faxne	4	2494.8	0.79797	0.8711

[1] di = disease severity score; fa = tree family; ne = needle age

The fungal communities from trees in each of the four disease classes do not form distinct groups in the canonical analysis of principal co-ordinates (Figure 1), though healthy and moderately healthy (classes 1 and 2) are separate from severely diseased trees (classes 3 and 4). There is considerable overlap between disease classes 1 and 2, and between disease classes 3 and 4. This analysis shows that healthy and moderately healthy trees are separated from diseased and severely diseased trees based on their fungal communities. Teratosphaeriaceae sp. 3, Teratosphaeriaceae sp. 13, Teratosphaeriaceae sp. 24 and *Lophodermium pinastri* were the dominant OTUs more strongly associated with unhealthy trees. In healthy trees Ascomycete sp. 2, *Phaeotheca fissurella*, Pleomassariaceae sp. *Dothistroma septosporum*, and Teratosphaeriaceae sp. 4 were the dominant OTUs within the fungal community.

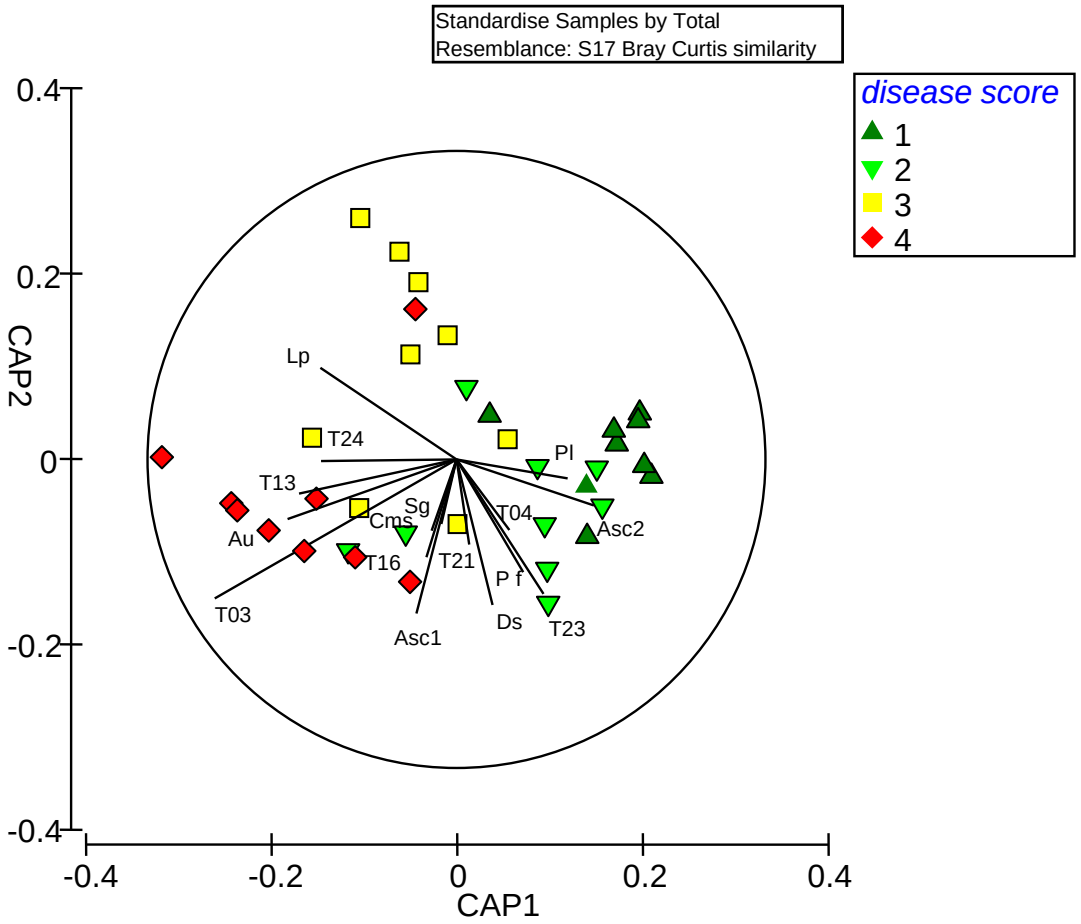

Fig.1. CAP of fungal communities in 36 pooled samples from different disease categories. The sixteen strongest vectors are included in the graph.OTUs depicted as vectors are (clockwise from top): Pl, Pleomassariaceae sp. ; Asc2, Ascomycete sp. 2; T04, Teratosphaeriaceae sp. 04; T23, Teratosphaeriaceae sp. 23; *Pf, Phaeotheca fissurella; Ds, Dothistroma septosporum;* T21, Teratosphaeriaceae sp. 21; Asc1, Ascomycete sp. 1; T16, Teratosphaeriaceae sp. 16; Cms, *Cyclaneusma minus* 'simile'; Sg, *Strasseria geniculata;*T03, Teratosphaeriaceae sp. 03; *Au, Aspergillus ustus;* T13, Teratosphaeriaceae sp. 13; T24, Teratosphaeriaceae sp. 24; *Lp, Lophodermium pinastri*.

V.4. DISCUSSION

V.4.1. Operational Taxonomic Units detected in Oonah plantation

This study, based on a single location and trees of a uniform age from three familes, detected 65 fungal OTUs. As the objective was to characterise fungi with the potential to significantly affect plantation health, either as pathogens or endophytes, the sampling strategy was calculated to capture the dominant fungi rather than to estimate the total biodiversity. The strategy of pooling PCR products before cloning and of selecting only 32 clones from each cloning reaction was adopted to reduce the number of 'singleton' species that contribute little to analyses such as (Howard and Robinson 1995). Pooling of PCR products before cloning has proven to be efficient for detection of most common ectomycorrhizal fungi in root tip samples (Morris et al. 2008, Morris et al. 2009).

Almost half of the OTUs detected in this study (29) were found in only a single pooled sample, indicating that needle fungal communities may be highly variable among trees at a single site. In contrast, 22 OTUs were detected in over 10% of the samples. These included four fungi, *Lophodermium pinastri*, *Cyclaneusma minus*, *Strasseria geniculata* and *Dothistroma septosporum* previously reported as common pathogens or endophytes in *Pinus radiata* in Tasmania (Podger 1984, Podger and Wardlaw 1990a).

This study indicates that Teratosphaeriaceae may represent a hither to overlooked component of pine needle fungal communities, with 18 species detected. These species are well known to be very slow growing in culture, small and dark coloured and could easily be overlooked or outcompeted by faster growing fungi in studies relying on fungal isolation. The most common species that have been systematically isolated and identified from *P. radiata* needles are those that grow relatively quickly in culture and are easily recognisable e.g. *Lophodermium pinastri* (Choi and Simpson 1991, Ridley and Dick

2001), *Cyclaneusma minus* and species of *Sporormiella* (Botes et al. 1997). Teratosphaeriaceae has only recently been separated from Mycosphaerellaceae (Crous et al. 2007) and unidentified species of Mycosphaerellaceae were also detected by DNA analysis in *Pinus taeda* (Arnold et al. 2007).

V.4.2. Association of needle cast pathogens with SNC

Cyclaneusma minus, *L. pinastri* and *S. geniculata* have been previously considered to be potential causal agents of SNC (Podger and Wardlaw 1990a), though only *L. pinastri* showed any correlation with disease severity in this study. *Cyclaneusma minus* 'simile' or 'verum' were equally present in all disease classes or present in only a small number of samples.

Podger and Wardlaw (1990a stated that *C. minus, L. pinastri* and *S. geniculata* were ubiquitous on fallen dead needles, but none was consistently associated with recently cast needles from SNC-affected trees. It is however possible, as suggested by Podger and Wardlaw (1990a), that at other sites in Tasmania any one or a combination of the three pathogens could cause SNC. Since our study showed that there was a trend for the different needle pathogens to be more prevalent on specific families, the predominant fungal pathogen(s) at a site might also depend upon the host genotype, though *L. pinastri* was also frequently detected and isolated from green, attached, needles of *P. radiata* at other Tasmanian plantation sites (Prihatini et al. 2014b) while *C. minus* and *S. geniculata* were less frequently detected. Though *C. minus* was detected at moderate frequency in the current study, its frequency of detection was correlated only to host family and not to disease class or needle age.

Trees with contrasting levels of SNC severity have significantly different fungal communities, but family pedigree and different age of needle are not clearly distinguished by their fungal communities. While all common fungal pathogens previously associated with SNC were identified from this study, only *L. pinastri* was correlated with high levels of disease. Minter et al. (1978) showed that *P. sylvestris* is a host for four different *Lophodermium* species that had previously been lumped under *L. pinastri.* Of the four species, only *L. seditiosum* was considered a pathogen (Minter and Millar 1980). Kowalski (1982) isolated *L. pinastri* from 12% of living needles (particularly older needles) on 8-11 year old *P. sylvestris* and raised the hypothesis that *Lophodermium* needle cast is caused by a fungal community rather than a fungal species. Diwani and Millar (1987) determined that *L. pinastri* was saprophytic in fallen needles as their attempts to inoculate needles of *P. sylvestris* were unsuccessful, but Osono and Hirose (2011) showed that *L. pinastri* did not colonise fallen needles of *P. thunbergia* and *P. densiflora* on the forest floor but was already present in needles before they fell. Since the 1980s most of the research on *Lophodermium* needle-cast has focussed on *L. seditiosum;* e.g. Hanso and Drenkhan (2012) used a needle trace method to link historical outbreaks of needlecast, presumably caused by *L. seditiosum,* with high precipitation in the preceding summer.

Further investigations into the relationship between *L. pinastri* and SNC in *P. radiata* are recommended. No detrimental effects of *L. pinastri* are known from New Zealand, but New Zealand isolates of *L. pinastri* may represent a different species to that in Europe and Tasmania (Johnston et al. 2003, Prihatini et al. 2014b). Tasmanian isolates had over 99% ITS sequence similarity to ATCC28347, designated by Minter et al. (1978) as *L. pinastri* when *L. seditiosum* was first described, where as the New Zealand isolates had less than 95% similarity (Prihatini et al. 2014b). A taxonomic study including

multigene phylogenetic analyses would assist in determining whether or not *L. pinastri* still consists of more than one species and may assist in clarifying the role of *L. pinastri* in needle cast diseases in *P. radiata.* The interaction of fungal endophytes and parasites with environmental factors should also be considered. Spring needle cast is more prevalent in high, wet sites (Podger and Wardlaw 1990a) and the proportion of *P. sylvestris* needles colonised by *L. pinastri* increases at higher altitudes (Van Maanen et al. 2000).

V.4.3. Other fungi associated with SNC

Additional species that warrant further investigation include several Teratosphaeriaceae species; Teratosphaeriaceae sp. 23 was detected in almost all of the needle samples and is presumably endophytic as it was associated with needles form healthy trees as well as those affected by SNC. Teratosphaeriaceae sp. 03, in contrast, was detected most frequently in needles of disease class 4 and not at all in disease class 1. Targeted attempts to isolate species of Teratosphaeriaceae from *P. radiata* needles are recommended as the first step towards a greater understanding of the role of these species in tree health. The possibility that these fungi were detected from superficial needle contamination by spores from, e.g. nearby eucalypts, must be considered, but the high detection rate of Teratosphaeriaceae sp. 23 and the failure to detect the common eucalypt-infecting species such as *T. cryptic* or *T. nubilosa* indicates that this is unlikely.

The fungal communities associated with the different classes of SNC severity at Oonah were dissimilar. The involvement of multiple species in maintaining healthy trees or in causing disease cannot be ruled out e.g. an interaction between apotential pathogen such as *L. pinastri* or Teratosphaeriaceae sp. 3 and the presence or absence of other endophytic fungi. Other studies of fungal endophyte communities in healthy and unhealthy

trees, conducted using isolation methods, also revealed that several species of fungi were frequently isolated from healthy but not declining trees. *Xylaria* sp. was isolated frequently in healthy *Heisteria concinna* (Arnold et al. 2003) and several species of Xylariaceae have been more frequently found in healthy *P. radiata* (Ganley et al. 2008). The role and identification of the other dominant endophytic fungi associated with diseased and healthy *P. radiata* trees should be further analysed, especially those that could have a role in the suppression of pathogenic needle fungi.

VI. NEEDLE FUNGI IN TASMANIAN *PINUS RADIATA* PLANTATIONS IN RELATION TO ELEVATION AND RAINFALL

Abstract

Needle fungi in conifers have been extensively studied to explore their diversity, but environmental factors influencing the composition of fungal communities in *Pinus radiata* needles have received little attention. This study was conducted to examine the influence of environment as defined by rainfall, elevation and temperature on the composition of fungal communities in pine needles just prior to the age at which spring needle cast (SNC) is observed. Needle samples were collected from 5-year-old *P. radiata* trees from 12 plantations in Tasmania. Interpolated data for the climate variables, including seasonal components for rainfall and temperature, were obtained from an enhanced climate data bank. Pooled needle samples were examined for the fungi they contained using DNA sequencing of cloned PCR products. Clones were grouped into operational taxonomic units (OTUs), and identified to their lowest possible taxonomic level by comparison with reference isolates and public DNA databases. There was considerable variation in needle fungal communities as revealed by DNA sequencing, with total annual rainfall and needle age showing a significant correlation with the fungal community composition. Needle fungi that have been previously associated with pathogenic behaviour, such as species of *Dothistroma*, *Strasseria*, *Cyclaneusma* and *Lophodermium*, were identified from this study but neither *Dothistroma septosporum* nor *Strasseria geniculata* were found at the two sites where SNC was later reported to be at a serious level. *Cyclaneusma minus* was present at one of those two sites, but absent at the other. In contrast, *Lophodermium pinastri* was an almost ubiquitous needle fungus. A possible significant finding of the present study is the large number of species of the Teratosphaeriaceae that were identified from *P. radiata* needles by their DNA sequences. Spring needle cast may be triggered by a stress that occurs under wet and cold conditions. The present study, conducted in plantations of an age that predates the expression of SNC, showed that *L. pinastri* was ubiquitous in needles at all sites at which the climate is appropriate for stressful conditions to develop. One species, labelled Teratosphaeriaceae sp. 03, was present at only three of the 12 sites, but serious SNC subsequently developed at two of these sites. A plausible scenario is that the

causal agent of SNC is *L. pinastri* acting in conjunction with Teratosphaeriaceae sp. 03 when stressful conditions are present.

VI.1. INTRODUCTION

As described in previous studies (Ganley and Newcombe 2006, Terhonen et al. 2011), Koukol et al. (2012), the *Pinus* phyllosphere is a rich and varied microbial community. Its composition is strongly influenced by both genotypic and environmental factors (Botes et al. 1997, Van Maanen et al. 2000, Wang and Guo 2007). These factors can affect the complex interactions between microbial organisms, which in turn influence their interactions with their host plant (Giauque and Hawkes 2013), e.g. such interactions may trigger a commensal or mutualist fungal species to act as a more virulent parasite (Krabel et al. 2013). Abiotic or environmental factors influencing the composition of fungal communities associated with *P. radiata* needles have received little attention.

Studies have shown that endophyte communities in *P. sylvestris* needles can vary according to location (Gourbiere and Debouzie 2003) and altitude (Gourbiere et al. 2001). Communities in *Picea mariana* needles have been reported to vary with latitude (Sokolski et al. 2007). A study by Hata & Futai (1996) showed that endophytic mycoflora associated with 45 pine species or varieties were similar for those pines belonging to the same taxonomic group. That study also concluded that endophytic colonization might reflect the degree of adaptation of the host to local biotic and abiotic factors. Endophytes appear to play a role in protecting trees from pathogens (Arnold et al. 2003, Bradshaw 2004, Gao et al. 2010).

Spring needle cast (SNC) in Tasmania appears in young pine plantations at canopy closure (age 6-7 years) and is manifested by the rapid browning of one-year-old needles followed by the heavy casting of needles. Spring needle cast is not considered to be a classical needle blight disease caused by a primary fungal pathogen (Podger and Wardlaw 1990a); instead, it is thought to be due to a suite of endophytic fungi that are triggered into secondary pathogenic activity by an environmental stress. In their study of 53 stands selected for climatic analysis, (Podger and Wardlaw 1990a) found that 23 of the sites were assessed as having SNC, and that these stands were restricted to a mean annual rainfall range of 1200–2000 mm and mean annual temperature range 8–11.5°C. Although *Cyclaneusma minus*, *Lophodermium pinastri* and *Strasseria geniculata* have been putatively associated with the disease in Tasmania, there were many areas with severe SNC where Podger and Wardlaw (1990a) found no fungal fruiting bodies on attached needles, dead or alive, but these three species of fungi were ubiquitous on fallen needles at sites both affected and unaffected by SNC.

The present study was conducted to explore the needle fungi present in 5-year-old *P. radiata* in Tasmania just prior to the age at which SNC is observed, and to examine the influence of environment as defined by rainfall, altitude and temperature on the composition of fungal communities.

VI.2. METHODS AND METHODS

VI.2.1. Sampling sites and environmental data

12 plantations for the collection of needle samples from 5-year-old *P. radiata* trees in Tasmania were chosen (Figure 1). It was originally planned to have three replications

(sampling sites) of each of the four factorial combinations of altitude and mean annual rainfall, with altitude dichotomised using 320 m as the cut-point, and rainfall dichotomised using 900 mm as the cut-point. For rainfall, however, it was later realised that this would result in a poor separation between the wettest of the ‘dry’ sites and the driest of the ‘wet’ sites. A more meaningful separation of mean annual rainfall was obtained by splitting the sites into three groups, using 800 mm as the upper cut-point of the ‘dry’ sites, 1100 mm as the lower cut-point of the ‘wet’ sites, and having an intermediate category of sites with rainfall in the range 800–1100 mm (see Table 1). Interpolated estimates (‘data drill’) of various aspects of rainfall and temperature data were downloaded from SILO (an enhanced climate data bank; (Queensland Government 2014): these included average annual mean temperature, average annual minimum and maximum temperature, average winter minimum and maximum temperature, average annual rainfall, and total monthly rainfall in winter and in summer. At each site, five 20-tree plots had been established for a previous study (unpublished), and needle samples were obtained from those plots.

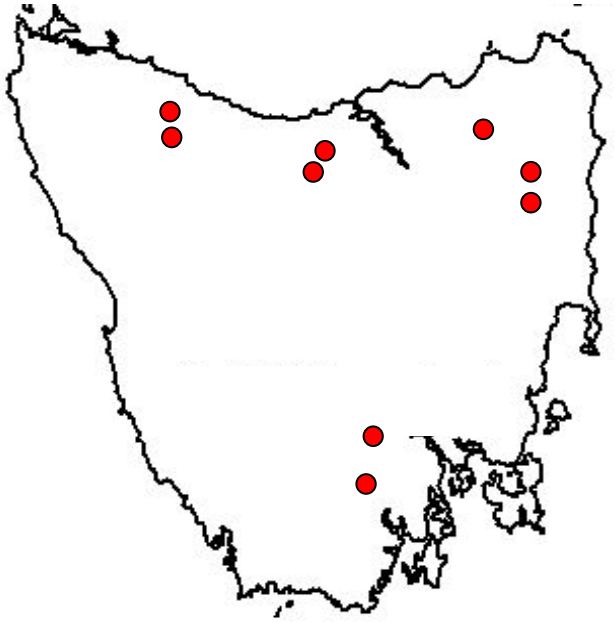

Figure 1. Location of pine plantations in Tasmania from which materials were collected (latitude, longitude and other information of these sites is presented in Table 1).

Table 1. Location (latitude and longitude), altitude and annual rainfall for each site. Inglis River and Oonah were reported to have SNC, while other sites were assessed as having no sign of, or low levels of, SNC after sample collection in the present study.

		Lat-long (Ave from GPS)		Altitude	Annual rainfall	AvTmin	AvTminW	TotRnW
Site	Coupe	North	East	(*)m	(**)mm	(***)^{o}C	(***)^{o}C	(***)mm
Branches Creek	BC029A	41.2653	146.6629	131 (L)	744 (D)	8.6	5.2	87.7
Franklin	FN014H	43.0573	146.8787	293 (L)	1123 (W)	5.3	2.6	115.2
Inglis River	IR035D	41.1087	145.5974	111 (L)	1353 (W)	7	4.2	170.8
Long Hill	LH106A	41.3426	146.4901	120 (L)	988 (M)	6.9	3.7	131.8
Nicholas 1	NI162B	41.4511	147.9764	338 (H)	915 (M)	5.7	2.2	82.9
Nicholas 2	NI166A	41.4719	147.9829	324 (H)	915 (M)	5.7	2.2	82.9
Oonah	OO072B	41.2299	145.6152	454 (H)	1439 (W)	6.9	4.1	177.5
Plenty	PL020X	42.8659	146.8929	427 (H)	876 (M)	4.7	2.0	85.2
Springfield 1	SF121F	41.2118	147.6107	311 (L)	785(D)	8.1	4.8	89.4
Springfield 2	SF121B	41.2114	147.6261	294 (L)	785 (D)	8.1	4.8	89.4
Styx	SX038Z	42.7770	146.8269	539 (H)	714 (D)	5.6	2.1	74.1
Tower Hill	TH135A	41.5277	147.9119	512 (H)	716 (D)	6	2.3	66.9

* Sites with altitude below 320 m above sea level are categorised as low (L), and sites with altitude at or above 320 m are categorised as high (H).

** Average of annual rainfall for the years 2000–2008 (samples were collected in 2008). Sites with mean annual rainfall below 800 mm are categorised as dry (D), sites with mean annual rainfall above 1100 mm are categorised as wet (W), and sites with mean annual rainfall between these extremes are categorised as moderate (M).

*** Some additional climate data downloaded from SILO (Queensland Government 2014), viz. average annual minimum temperature(AvTmin), average winter minimum temperature (AvTminW) and total rainfall per winter month (TotRnW),are also shown in this table, whereas some other environmental data used in the statistical analyses (e.g.average annual mean temperature, total rainfall in summer, total rainfall in winter, etc.) are not presented here.

VI.2.2. Needle samples

Four composite needle samples were obtained from each site, corresponding to needles of four different age classes (1-year-old, 2-year-old, 3-year-old and fallen needles). Five trees were randomly selected from each of the five 20-tree plots and a single fascicle of each class was taken from each tree. Fascicles in the same class were pooled for each site, placed into paper bags and stored at 10°C for 1-2 days before drying at 42°C.

VI.2.3. DNA extraction

Approximately 1cm was cut from the middle part of each needle, placed into a 1.5mL microcentrifuge tube and stored at -80^{o}C prior to grinding with a mortar and pestle. DNA extractions were performed using the procedure described in Glen et al. (2002).

VI.2.4. Polymerase Chain Reaction (PCR) and electrophoresis

To amplify the ITS region, PCR was carried out using primers ITS4A(Larena et al. 1999)and ITS5 (White et al. 1990)with the conditions as described in Prihatini et al. (2014b**).** PCR products that were clearly visible on an ethidium bromide stained agarose gel were cloned directly after purification with an UltraClean® PCR Clean-up Kit (MO BIO Laboratories, USA). Samples with no product after the first round of PCR were diluted 1 in 5 and re-amplified by nested PCR using the primers ITS1 and ITS4 (White et al. 1990). Nested PCR products were purified and cloned as above.

PCR using ITS4A and ITS5 was not successful for all of the 48 pooled samples. Fifteen samples with no visible amplification product were re-amplified by nested PCR and fragments visible on an agarose gel were obtained. All 48 samples obtained by standard PCR or nested PCR were cloned.

VI.2.5. Cloning of amplified DNA, screening and DNA sequencing

The PCR and nested PCR products were cloned using pGEM®-T Vector Systems (Promega) according to the technical manual provided. Clones were screened by restriction fragment length polymorphisms (PCR-RFLP) and 3 or 4 clones from each RFLP group from each needle sample were sequenced. Chromatograms were viewed in ChromasPro version 1.34 software and edited to remove poor quality sequences at each end; the sequence was then saved in FASTA format. Sequencing of PCR products was performed by Macrogen Inc. (Seoul, Korea).

VI.2.6. Fungal DNA identification

A basic local alignment search tool (BLAST, Altschul et al. 1990) search of public DNA databases retrieved sequences of high similarity. Sequences were grouped according to BLAST search results, and aligned with sequences of high similarity using ClustalW (Bultman et al. 2004). If single nucleotide polymorphisms occurred between clones from the same OTU, chromatograms were rechecked to confirm these. Isolates or clones with less than 2% sequence variation were grouped into operational taxonomic units (OTUs). The OTUs were identified to the lowest possible taxonomic level based on sequence similarity to known fungi from public DNA databases and reference cultures (Prihatini et al. 2014b). Phylogenetic analysis helped to refine the discrimination and identification of OTUs. Several sequences were selected from the best matches retrieved by BLAST searches of public databases for each OTU and included in phylogenetic analysis for that OTU. One or two sequences from more distantly related taxa were also included as outgroups. All sequences for each OTU were aligned using the ClustalW program in BioEdit version 7.0.9.0 (Hall 1999) prior to phylogenetic analysis using DNAml from the

PHYLIP package (Felsenstein 1989). Trees were drawn by TreeView software (Page 1996) and edited using MEGA4 software (Tamura et al. 2007).

VI.2.7. Statistical analysis

All statistical analyses to study the community of needle fungi present were conducted using PRIMER v6 (Clarke and Gorley 2006), with its add-on PERMANOVA+ v1.0.2 (Anderson et al. 2008). The basic data matrix consisted of the presence or absence of 71 ascomycetous OTUs discriminated from the phylogenetic analysis on the 34 pooled samples that yielded clones. A Bray-Curtis resemblance (similarity) matrix constructed from all possible pairs of samples was the input for each of the routines used. Both permutational multivariate analysis of variance (PERMANOVA)and canonical analysis of principal coordinates (CAP) was used to test separately whether the factors needle age, altitude(low vs. high) and total annual rainfall (dry, intermediate, wet) produced significantly different fungal assemblages. The advantage of CAP is that it is specifically designed to find canonical axes that maximise differences among pre-defined groups in the data cloud, and it also tests individual samples for the correctness or otherwise of their group membership by cross-validation, employing a leave-one-out procedure. The third routine used, DistLM, modelled whether the climate variables altitude, rainfall and temperature could explain a significant proportion of the variation in the data cloud. Because the rainfall and temperature data were available in several different forms (e.g. temperature was expressed in seven different guises as overall average annual temperature, average annual minimum temperature, average annual maximum temperature, average winter minimum temperature, average winter maximum temperature, average spring minimum temperature and average spring maximum temperature, and rainfall was

expressed as total annual rainfall, total rainfall per winter month and total rainfall per spring month) and there were high correlations amongst them, principal component analysis (PCA) was first applied to the raw data, standardised to have unit variance. Subsequently, the DistLM modelling was conducted on the first three principal components, which accounted for almost all of the total variation. For DistLM, the Bayesian selection criterion BIC was used as a 'stopping rule'. All three routines of the PERMANOVA+ program that were used here employed 9999 permutations.

VI.3. RESULTS

VI.3.1. Identification of fungi amplified from *P. radiata* needles

A total of 504 clones were sequenced but only 439 clones gave legible sequence results, allowing OTU assignment and identification. Overall, 407 Ascomycetes sequences were identified. Sequences were grouped into OTUs with over 98% sequence similarity. After phylogenetic analysis, 70 ascomycetes OTUs were discriminated, of which 43 were detected from more than one sample (Table 2). The last 15 entries in Table 2 contain 14 Basidiomycetes and a lichen-forming ascomycete. These are included in the list of OTUs for the sake of completeness but were excluded from the statistical analyses as they were deemed unlikely to play a causal role in SNC or related diseases.

Table 2. List of all OTUs observed in this study with the number of cloning samples in which each OTU was present and a GenBank sequence accession representative of that OTU.

Class;Order; Family	OTUs	No. of cloning samples	GenBank Accession
Dothideomycetes; Botryosphaeriales			
Incertaesedis	Botryosphaeriales sp. 2	2	KM216352
	Botryosphaeriales sp. 3	1	KM216350
Dothideomycetes; Capnodiales			
Davidiellaceae	*Davidiella* sp.	7	KM216336
	Davidiellaceae sp. 1	3	KM216333
	Davidiellaceae sp. 2	1	KM216347
Incertaesedis	Capnodiales sp. 2	1	KJ406757
	Capnodiales sp. 3	1	KM216329
	Phaeotheca fissurella	3	KM216349
Mycosphaerellaceae	*Dothistroma septosporum*	2	KJ406805
	Mycosphaerellaceae sp. 1	3	KJ406789
	Phaeothecoidea sp. 1	4	KJ406802
	Phaeothecoidea sp. 2	1	KJ406792
	Pseudocercospora sp. 1	1	KJ406795
	Ramularia stellenboschensis	1	KJ406791
Teratosphaeriacae	*Catenulostroma* sp.	15	KJ406761
	Teratosphaeria associata	2	KJ406767
	Teratosphaeria capensis	1	KJ406771
	Teratosphaeria parva	1	KJ406775
	Teratosphaeria suttonii	1	KJ406763
	Teratosphaeriaceae sp. 03	3	KJ406765
	Teratosphaeriaceae sp. 04	1	KM216332
	Teratosphaeriaceae sp. 05	2	KJ406766
	Teratosphaeriaceae sp. 06	2	KJ406768
	Teratosphaeriaceae sp. 07	1	KJ406760
	Teratosphaeriaceae sp. 08	2	KJ406759
	Teratosphaeriaceae sp. 09	1	KJ406770
	Teratosphaeriaceae sp. 10	3	KJ406764
	Teratosphaeriaceae sp. 14	4	KJ406758
	Teratosphaeriaceae sp. 16	1	KJ406786
	Teratosphaeriaceae sp. 17	1	KJ406785
	Teratosphaeriaceae sp. 18	2	KJ406787
	Teratosphaeriaceae sp. 19	1	KM216331
	Teratosphaeriaceae sp. 21	2	KJ406769
	Teratosphaeriaceae sp. 23	10	KJ406784

Class;Order; Family	OTUs	No. of cloning samples	GenBank Accession
Dothideomycetes;Dothideales			
Dothioraceae	*Aureobasidium pullulans*	2	KJ406824
	Sydowia polyspora	1	KJ406826
Incertaesedis	Dothideales sp.	10	KJ406831
Dothideomycetes; Pleosporales			
Didymellaceae	*Leptosphaerulina* sp.	1	KJ406837
Incertae sedis	Pleosporales sp.	2	KJ406836
	Didymellaceae sp. 1	1	KJ406839
Montagnulaceae	*Paraphaeosphaeria michotii*	3	KJ406846
Phaeosphaeriaceae	*Phaeosphaeria* sp. 3	1	KJ406845
Sporormiaceae	*Sporormiella intermedia*	2	KJ406862
	Sporormiella sp. 3	1	KJ406867
Eurotiomycetes; Chaetothyriales			
Herpotrichiellaceae	*Exophiala eucalyptorum*	2	KJ406880
Incertae sedis	Chaetothyriales sp. 1	3	KJ406877
	Chaetothyriales sp. 2	4	KJ406870
	Chaetothyriales sp. 3	1	KJ406884
Eurotiomycetes; Eurotiales			
Trichocomaceae	*Penicillium* aff. *chrysogenum*	1	KJ406888
	Penicillium corylophilum	4	KJ406887
	Penicillium sp.	1	KJ406895
	Penicillium namyslowskii	1	KJ406890
Lecanoromycetes; Lecanorales			
Incertaesedis	Lecanorales sp.	1	KM624596
Leotiomycetes; Helotiales			
Helotiaceae	*Varicosporium elodeae*	2	KM216334
Incertae sedis	Helotiales sp. 4	1	KM216351
Phacidiaceae	*Ceuthospora pinastri*	2	KJ406926
Sclerotiniaceae	*Torrendiella eucalypti*	2	KM216355
Leotiomycetes; Incertae sedis			
Incertaesedis	*Cyclaneusma minus* 'simile'	8	KJ406921
	Cyclaneusma minus 'verum'	9	KJ406905
	Meliniomyces sp.	1	KM216335
Leotiomycetes; Rhytismatales			
Incertaesedis	*Fulvoflamma* sp.	1	KM216341
	Rhytismatales sp. 1	3	KJ406966
	Rhytismatales sp. 4	2	KJ406964
Rhytismataceae	*Lophodermium conigenum*	4	KJ406944
	Lophodermium pinastri	14	KJ406949
Sordariomycetes; Sordariales			
Incertae sedis	Sordariales sp.	1	KJ406980
Lasiosphaeriaceae	*Bagadiella* sp.	1	KM216344

Class;Order; Family	OTUs	No. of cloning samples	GenBank Accession
Sordariomycetes; Xylariales			
Amphisphaeriaceae	*Pestalotiopsis maculiformans*	1	KJ406982
Ascomycetes of uncertain taxonomic position			
	Ascomycete sp. 1	3	KM216353
	Ascomycete sp. 3	1	KM216354
	Strasseria geniculata	1	KM216342
Agaricomycetes; Agaricales			
Physalacriaceae	*Armillaria luteobubalina*	2	KM624597
Strophariaceae	*Pholiota multicingulata*	2	
Agaricomycetes; Boletales			
Serpulaceae	*Serpula himantioides*	1	KM216346
Tremellomycetes; Tremellales			
Tremellaceae	*Cryptococcus* aff. *amylolyticus*	1	KM216348
	Cryptococcus sp. 1	1	KM216340
	Cryptococcus sp. 2	1	KM216339
	Cryptococcus victoriae	2	KM216343
	Tremella sp.	2	KM216338
	Tremellaceae sp. 1	1	KM624601
	Tremellaceaesp. 2	1	KM624604
Incertaesedis	Basidiomycete sp. 1	1	KM624603
	Basidiomycete sp. 2	2	KM624606
	Basidiomycete sp. 7	2	KM624589

VI.3.2. Fungal prevalence across sites

Clones for DNA identification were obtained for only 34 out of the 48 pooled samples. However, all 12 plantation sites were represented by at least one pooled sample that gave rise to clones. Each OTU observed in this study (Table 2) was scored as present or absent in each pooled sample. Fungal OTUs present in at least 3 of the 34 pooled samples are presented in Table 3. Forty-one OTUs were identified only from a single site but 7 of these were detected in more than one cloning sample at that site. The remaining 30 OTUs were present in more than one site, but only 6 OTUs occurred at 6 or more of the sites. Several of the commonly found OTUs were well-known pine associates belonging to the genera *Catenulostroma*, *Lophodermium* and *Cyclaneusma*, but the distribution of pathogenic

associates varied among the 12 plantation sites, and none was found at all 12 sites. The two most dominant OTUs were *Catenulostroma* sp. found in 15 pooled samples at 7 sites, and *Lophodermium pinastri*, found in 14 pooled samples and at all but one of the 12 sites. *Cyclaneusma minus* 'simile and *C. minus* 'verum' were each identified at half of the sites. *Strasseria geniculata* and *Dothistroma septosporum* were each only found at a single site, but are included in Table 3 because of their putative previous involvement as causal agents of needle cast disease.

Table 3. The major OTUs present in 12 young *P. radiata* plantations in Tasmania listed in decreasing order of prevalence in terms of number of pooled samples.

*Site (No. of pooled samples)	ST (3)	TH (4)	PL (3)	N1 (3)	N2 (3)	ON (2)	SP1 (3)	SP2 (4)	BC(2)	LH (2)	FR (4)	IR (1)
**Environmental conditions	HD	HD	HM	HM	HM	HW	LD	LD	LD	LM	LW	LW
***OTUs:												
Catenulostroma sp.	2	3	0	2	3	0	0	2	0	2	0	1
Lophodermium pinastri	0	1	1	1	1	1	2	2	1	1	2	1
Sydowia polyspora	0	2	0	1	0	1	1	1	0	1	2	1
Teratosphaeriaceae sp. 23	1	0	0	0	1	0	3	3	0	0	2	0
Cyclaneusma minus 'verum'	0	3	0	1	1	0	0	1	0	2	0	1
C. minus 'simile'	0	2	0	1	0	0	0	2	1	0	1	1
Davidiella sp.	1	2	1	1	1	0	1	0	0	0	0	0
Penicilliumc orylophilum	0	0	2	0	0	0	1	0	0	0	1	0
L. aff.*conigenum*	0	0	0	0	1	0	0	0	0	0	2	1
Chaetothyriales sp. 2	0	0	1	0	0	0	1	1	0	1	0	0
Phaeothecoidea sp. 1	0	0	2	1	0	0	0	0	0	1	0	0
Teratosphaeriaceae sp. 14	0	0	0	0	0	0	3	0	0	0	1	0
Ascomycete sp. 1	0	0	0	2	0	0	1	0	0	0	0	0
Chaetothyriales sp. 1	0	0	0	0	0	1	0	1	0	0	0	1
Davidiellaceae sp. 1	0	2	0	0	0	0	1	0	0	0	0	0
Mycosphaerellaceae sp. 1	0	0	0	0	0	0	0	1	0	1	1	0
Paraphaeosphaeriamichotii	0	0	0	0	0	1	1	0	0	0	1	0
Phaeotheca fissurella	0	0	1	0	0	0	0	2	0	0	0	0
Rhytismatales sp. 1	0	0	0	1	0	0	0	0	0	0	2	0
Teratosphaeriaceae sp. 3	0	0	0	0	0	1	0	0	0	1	0	1
Teratosphaeriaceae sp. 10	0	0	0	0	0	1	1	1	0	0	0	0
Dothistroma septosporum	0	0	0	0	0	0	0	2	0	0	0	0
Strasseria geniculata	0	1	0	0	0	0	0	0	0	0	0	0

*ST= Styx; TH= Tower Hill; PL= Plenty; N1= Nicholas 1; N2= Nicholas 2; ON= Oonah; SP1= Springfield 1; SP2= Springfield 2; BC= Branches Creek; LH= Long Hill; FR= Franklin; IR= Inglis River

**Environmental conditions were categorised as high (H) or low (L) altitude and dry (D), intermediate (M)or wet (W) based upon annual rainfall.

***Numbers in columns 2 to 13 indicate the number of pooled DNA samples in which the OTU was found at a particular site. OTUs in bold are fungal species that have been previously found to be associated with SNC or other needlecast diseases.

VI.3.3. Fungal communities

Permutational multivariate analysis of variance (PERMANOVA) produced a significant separation (P=0.0151) of the fungal communities present at the three levels of annual rainfall (<800 mm, dry; 800–1200 mm, intermediate; >1200 mm, wet). Use of CAP provided further evidence of the importance of rainfall, strongly separating the three groups (P=0.0008). In Figure 2, Axis 1is seen to separate the sites of intermediate rainfall from the other sites, whereas Axis 2 separates the wet sites from the others. PERMANOVA and CAP analyses of fungal communities provided only marginal, rather thanstrong, evidence for a significant association between communities and altitude (P=0.0583 and 0.0949, respectively). Similarly, using DistLM, no evidence was found that fungal communities have a significant correlation to temperature (P=0.262 using a composite score from PCA), whereas using a composite PCA score evidence was found of an association with rainfall (P=0.0158), thereby supporting the results from PERMANOVA and CAP that rainfall is the most significant environmental variable influencing fungal community assemblages.

Significant differences in fungal assemblages as a function of needle age were also obtained using permutational multivariate analysis of variance. From PERMANOVA, pairwise differences were indicated between 1-yr-old needles and fallen needles (P=0.0159) and between 2-yr-old needles and fallen needles (P=0.0184), with marginal

evidence of an assemblage difference between 3-yr-old needles and fallen needles (P=0.0725). CAP, which is an alternative way of testing whether the multivariate means are different for the four different age classes, was unequivocal in indicating a significant difference between age classes (P=0.0181 [trace test] or 0.006 [test based on first squared canonical correlation]), with 9 out of the 11 fallen needle samples being correctly classified. No 1-yr-old or 2-yr-old needle sample was correctly classified, and only 3 of the eight 3-yr-old needle samples were correctly classified.

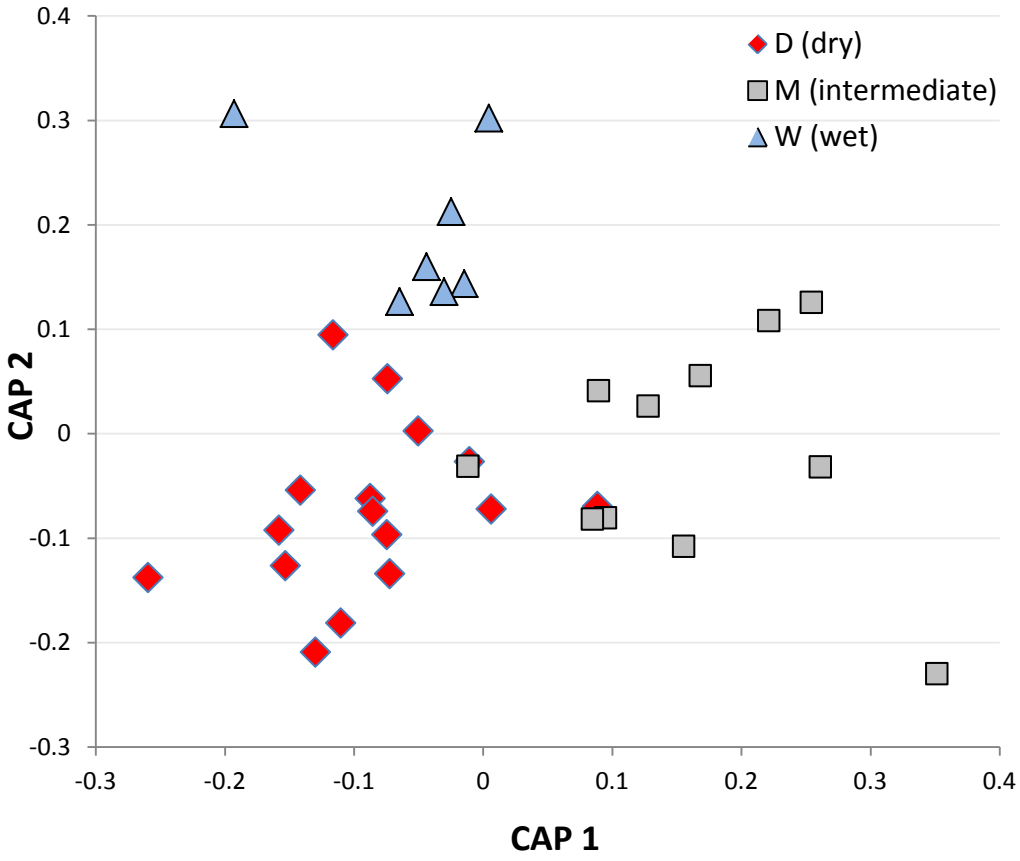

Figure 2.CAP analysis of fungal communities in 34 pooled DNA samples from 3 rainfall categories (dry, intermediate, wet) of *P. radiata* plantations in Tasmania.

This suggests that fallen needles have a different fungal assemblage to that of the three age classes, with their OTUs being more distinct than those in the needles retained by the trees. Nevertheless, there were some species, e.g. *Lophodermium pinastri*, which can be considered ubiquitous, as it was present in 14 of the cloning samples, 9 of which were in fallen needles. The sites at which *L. pinastri* was not present in the fallen needles sample were Tower Hill, Styx, and Branches Creek, the three driest sites.

VI.4. DISCUSSION

Needle fungal communities varied significantly between the 12 different plantation sites of 5 year old *P. radiata* that were investigated in Tasmania. Other studies also indicate similar significant variation in the needle fungal communities of another *Pinus* species (*P. sylvestris*) across different sites and locations in the Massif Central in France, colonized by the same or similar needle cast fungi active in Tasmania, viz. *Lophodermium pinastri* and *Cyclaneusma minus* (Gourbiere et al. 2001, Gourbiere and Debouzie 2003).

The present study found that temperature at a site, irrespective of whether it is an average, minimum or maximum temperature, does not strongly influence the composition of needle fungal communities. In contrast, statistical analysis using PERMANOVA, CAP, and DistLM of climate data after applying PCA to their standardised values, showed that rainfall was the key factor correlating with fungal assemblages at the 12 sites. In forest health surveys subsequent to this study (Wardlaw, unpublished data), only the two wettest of the 12 sites (Inglis River and Oonah) were reported to have a serious level of SNC. These two sites in northwest Tasmania are in the same general area where a previous study found other plantations that had SNC (Podger and Wardlaw 1990b). The average annual rainfall of these two wettest sites during the nine years preceding the survey, 1439 and

1353 mm, respectively (see Table 1), falls squarely within the predicted range of 1200-2000 mm that was determined by Podger & Wardlaw (Podger and Wardlaw 1990a) from their analysis of the climatic characteristics of the disease distribution.

In another study (Prihatini et al. 2014), a *Pinus radiata* marker aided selection trial planted in 1999 in Oonah, Tasmania (a locality also used in the present study but in a different, and younger, plantation), three replicates of pine needle samples from four different needle cast disease classes, three full-sib families with known breeding values for SNC resistance, and three different needle ages, for a total of 108 pooled samples, were collected in spring 2007 and had their needle fungal communities analysed in a similar manner to that in the present study. *Lophodermium pinastri*, *Dothistroma septosporum* and *Cyclaneusma minus* occurred frequently (12, 16 and 19 needle samples, respectively), but notably 17 species of hither to unknown members of the Teratosphaeriaceae were identified, of which Teratosphaeriaceae sp. 23 and Teratosphaeriaceae sp. 03 occurred in 33 and 12, respectively, of the 36 needle samples. Teratosphaeriaceae sp. 23, a presumed endophyte, was detected in healthy trees as well as those affected by SNC, but Teratosphaeriaceae sp. 03, in contrast, was detected most frequently in needles of the highest disease class and never in healthy needles. These Teratosphaeriacae species were detected only by DNA sequences and were not isolated in the present study, and, to our knowledge, have not previously been isolated.

Podger and Wardlaw (1990a) postulated that SNC was due to the pathogenic activity of one or a suite of pathogens that switched from an endophytic phase following an as yet unidentified but ephemeral stress. This endophyte may not necessarily be a primary pathogen but secondary to an environmental stress when conditions are cold and wet, the most likely candidate being *L. pinastri* due to its ubiquitous nature. Of the four

putative causal agents of SNC (*S. geniculata, C. minus*, *D. septosporum* and *L. pinastri*), only *L. pinastri* was strongly associated with needles of trees with SNC (Prihatini et al. 2014b) . It is suggested that this *Lophodermium* species is present throughout these plantations and if the right stress is there, it can act as a pathogen, perhaps in collaboration with Teratosphaeriaceae sp. 03.The latter species is not ubiquitous but seems to be present at the sites that will subsequently develop SNC and it also has an association with trees that have higher levels of SNC symptoms (Prihatini et al. 2014b).

VII. GENERAL DISCUSSION

Pinus radiata is one of the most important species of world forestry plantation and many pests and diseases have followed the expansion of this species around the world. *Cyclaneusma* needle cast is reported as one of the serious needle cast diseases of *P. radiata* plantations in New Zealand, caused by the fungus *Cyclaneusma minus* (Bulman, 1988; Blankenship et al., 2001; Bulman and Gadgil, 2001). Tasmanian *P. radiata* plantations suffer from a needle cast disease with similar symptoms known as SNC. The aetiology of SNC has long been a point of debate between Tasmania and New Zealand foresters (Wardlaw, personal communication). Unlike for New Zealand, *C. minus* is regarded as weak pathogen in Tasmania. Spring needle cast is considered to be caused by one or a suite of fungi that are triggered by an unknown environmental stress (Podger and Wardlaw 1990). Stands with moderate or severe SNC can be expected to suffer potential losses in clear-fall volume of 30-50% (Podger and Wardlaw 1990a). Three species of fungi have been putatively associated with SNC, i.e. *Lophodermium pinastri*, *Strasseria geniculata* and *C. minus* but the etiology of this potentially damaging disease is poorly understood.

Understanding endophyte diversity is crucial to determining the role of endophytes in forest health (Ganley et al 2008). Endophytes are being increasingly recognized and manipulated for their role in protecting their host plants from natural enemies such as insects (Jaramillo et al. 2009), nematodes (zum Felde et al. 2009) and pathogenic fungi (Ownley et al. 2010). Many pathogens, including serious pathogens of conifers, such as *D. septosporum* in *P. radiata* (Choi and Simpson 1991, Bradshaw 2004), *Sphaeropsis sapinea* in *P. nigra* and *P. sylvestris* (Flowers et al. 2003) and *Phaeocryptopus gaeumannii* in

Pseudotsuga menziesii (Morales et al. 2012) have a latent phase often considered as an endophytic stage (Sieber 2007). There have only been a limited number of investigations of needle fungal endophyte communities in *P. radiata*. Studies of *P. radiata* needle fungi in the southern hemisphere have focused on the study of single pathogens such as the distributions of pathogen *Sphaeropsis pinea* and *Diplodia scrobiculata* in South Africa (Burgess et al. 2004) and endophytes that offer the promise of biocontrol, for example the characterization of endophytic *Beauveria* as biological control agents against insect pests of pine in New Zealand (Zabalgogeazcoa 2008; Reay et al. 2010).

The main objective of this study was to identify needle fungi which are associated with both healthy and SNC-affected trees in *P. radiata* plantations in Tasmania. There were 4 main components to the study.

1. Identification of the fungal species –Moleuclar Operational Taxonomic Units (MOTUs) commonly associated with *P. radiata* needles in Tasmanian plantations.

A total of 156 MOTUs were recognised from isolates and DNA fragments amplified from pine needles. Generally in Pinaceae, needle fungal communities are dominated by Leotiomycetes followed by Dothidiomycetes (Arnold, 2007; Ganley, 2008b). However, the most frequently detected group in this study of P. radiata needles in Tasmania was Dothideomycetes. Leotiomycetes was the second most frequently detected group, by culturing as well as by DNA amplification. Sordariomycetes was the third most frequently detected group, but the most highly represented by isolation.

Only 10 of the 156 MOTUs were detected by direct DNA amplification and fungal isolation, 25 by isolation only, and the remaining 121 were detected by DNA only. The limitations associated with needle fungal surveys based on cultural techniques are

recognized and it is accepted that these limitations strongly influenced the fungi most frequently isolated from the different sites e.g. our failure to isolate the pathogen *Dothistroma septosporum* may be associated with its slow growth in culture and propensity to be overgrown by other fungal species. The sampling for isolations was also opportunistic and not designed to allow a robust comparison of frequency of occurrence among sites or with the MOTUs detected by the PCR and sequencing of needle DNA. Nonetheless, the substantial differences between the two datasets (OTUs and MOTUs) invite a comparison.

Both techniques detected a few commonly occurring, widespread species and many more that were detected infrequently or from a limited geographic range. This is consistent with fungal endophyte communities in other *Pinus* species (Martinez-Alvarez et al., 2012, Peršoh, 2013) other genera (Rajala et al., 2014) and indeed fungal communities in other substrates such as roots or soil (Danielsen et al., 2012). Most of the commonly isolated fungal species and many of the commonly detected MOTUs were consistent with previously known fungal associates of *P. radiata* or other pine or conifer species, e.g. species of *Lophodermium, Cyclaneusma* and *Coniochaeta*. PCR and sequencing, however, revealed a number of common endophytes not previously recorded from *P. radiata* e.g. members of the Teratosphaeriaceae.

No clear conclusions can be drawn for any of the fungi detected in this study and their relationship to tree health, though some interesting avenues for further inquiry have been indicated especially in respect to members of the Teratosphaeriaceae. More comprehensive metagenomic studies using next-generation sequencing will assist with developing our understanding of fungal endophytes, their lifestyles and host ranges. Targeted attempts to isolate and describe previously unknown members of the

Teratosphaeriaceae from pine needles will increase understanding of their roles in pines and other plants.

2. A multigene phylogeny study to determine the *Cyclaneusma* species present in Tasmania.

Two different morphotypes of *Cyclaneusma* cultures from Tasmania and mainland Australia were isolated for inclusion in the phylogenetic analysis of *Cyclaneusma*. The two clades of *C. minus* were consistent with the two different morphotypes of *C. minus* reported in a previous study (Bulman and Gadgil, 2001). One morphotype ('verum') was more closely related to *Cyclaneusma niveum* than to the other morphotype ('simile'). A formal description of the new species represented by the 'simile' clade is now required.

Confirmation of the occurrence of two *Cyclaneusma* species on *P. radiata* in Australia and New Zealand is a significant scientific step. Host tree responses to pathogens are variable and can be affected by several factors such as pathogen genetics, host genetics and environmental factors (Barrett et al., 2009). The molecular characterisation of the two *Cyclaneusma* species will facilitate examination of the role each plays in diseases such as CNC (Watt et al. 2012) and SNC (Podger and Wardlaw 1990) although, as discussed below, the research in this thesis has shown that *Cyclaneusma* does not appear to be associated with SNC. This study revealed a high genetic similarity of *Cyclaneusma* species in New Zealand and Tasmania therefore other factors such as host genetics, environmental factors or the presence/absence of endophytes may explain for the different responses of Tasmanian *P. radiata* trees to *Cyclaneusma* species in comparison with New Zealand. A recent publication (Watt et al. 2012) indicates that severity of CNC is highest in moderately warm, wet, and humid high-elevation environments located in the central North Island of New Zealand. Tasmanian conditions may prove too cold and dry for severe

CNC. Under climate change scenarios, it is projected that the potential distribution of another needle pathogen *D. septosporum* will expand southwards, with particular increases in climatic suitability in Tasmania where currently it is present but is not a pathogen that poses a severe problem due to colder conditions (Ireland et al. 2012, unpublished). Either of the two *Cyclaneusma* species might become a problem in Tasmania as climate changes. Environmental factors may also influence the interactions of needle pathogens with endophytes which may exert either a positive or negative influence on disease expression as indicated by the final two components of the thesis.

3. Investigation of the needle fungal communities (pathogens and endophytes) in *P. radiata* trees with varying levels of resistance to SNC.

Pinus radiata trees were scored for SNC damage in a resistance trial at Oonah in Tasmania. Pooled needle samples were examined for needle fungi using direct DNA sequencing of cloned PCR products. Clones were grouped into operational taxonomic units (OTUs), and identified to their lowest possible taxonomic level by comparison with reference isolates and public DNA databases. The very important advance offered by this study is that it was possible to identify the fungi present in living but diseased needles using molecular techniques. Original studies of SNC relied on the presence of sporulating structures on dead needles as evidence of the presence of a particular pathogen.

Sixty six OTUs of fungi were detected. The prevalence of each OTU in samples from different host families, needle ages and disease class was used as the basis for statistical analyses. Teratosphaeriaceae sp. 23 was the most commonly detected fungus and was present across all four disease classes, all three needle ages and all three families. *Cyclaneusma minus* ‘simile’ was the second most commonly detected fungus and was

present in approximately half of the samples equally across needle ages and disease classes. No specific fungi were consistently associated with young needles. *Lophodermium pinastri,* Teratosphaeriaceae sp. 3 and Teratosphaeriaceae sp. 13 were more commonly associated with trees in the higher disease classes. These same fungi were also found more frequently in older needles.

Cyclaneusma minus, L. pinastri and *S. geniculata* are three species that have been suggested as possible causal agents of SNC. While all three species were detected in the Oonah trial, only *L. pinastri* was detected at higher prevalence in higher disease classes than *Strasseria geniculata* and *C. minus* 'verum'. Though *C. minus* was detected at a moderate frequency in the current study, its frequency of detection was correlated to host family only and not to disease class or needle age.

The Oonah study showed that trees with contrasting levels of SNC severity have significantly different fungal communities. As such, healthy and moderately healthy trees could be separated from diseased and severely diseased trees based on their fungal communities. Seventeen species of hitherto unknown members of the Teratosphaeriaceae were identified. In particular, Teratosphaeriaceae sp. 3, Teratosphaeriaceae sp. 13, Teratosphaeriaceae sp. 24 and *L. pinastri* were the dominant OTUs more strongly associated with unhealthy trees. In healthy trees, Ascomycete sp. 2, *Phaeotheca fissurella,* Pleosporales sp. 1, *Dothistroma septosporum,* and Teratosphaeriaceae sp. 4 were the dominant OTUs within the fungal community.

The involvement of multiple species in maintaining healthy trees or in causing disease cannot be either ruled in or out by the results at Oonah e.g. an interaction between the pathogen *L. pinastri* and the presence or absence of other endophytic fungi. Further

investigations into the relationship between *L. pinastri*, member of the Teratosphaeriaceae and SNC in *P. radiata* are highly recommended.

4. Characterisation of the needle fungal communities associated with young radiata pine in plantations before the expression of SNC (including an investigation of the specific relationship between site environmental conditions as defined by rainfall and altitude, and the composition of fungal communities).

Needle samples were collected from 5-year-old *P. radiata* trees from 12 plantations across Tasmania. Interpolated data of climate variables, including seasonal components for rainfall and temperature, were obtained from an enhanced climate data bank. Pooled needle samples were examined for needle fungi using direct DNA sequencing of cloned PCR products. Clones were grouped into operational taxonomic units (OTUs), and identified to their lowest possible taxonomic level by comparison with reference isolates and public DNA databases. There was considerable variation in needle fungal communities as revealed by DNA sequencing.

Needle fungal communities present in 5-year-old *P. radiata* trees growing at different sites were strongly defined by the presence or absence of pine associates that are considered as 'true' endophytes such as species of the Teratosphaeriaceae. However, needle fungi that have been previously associated with pathogenic behaviour (species of *Dothistroma*, *Strasseria*, *Cyclaneusma* and *Lophodermium*) were also detected from young *P. radiata* trees but neither *D. septosporum* nor *S. geniculata* were found at the two sites where SNC was later reported to be at a serious level. *Cyclaneusma minus* was present at site one only. In contrast, *L. pinastri* was an almost ubiquitous needle fungus. These results

support findings from the Oonah trial i.e. *L. pinastri* is more closely associated with SNC than *C. minus* and *S. geniculata.*

The study found that the composition of needle fungal communities was unrelated to temperature, irrespective of whether it is an average, minimum or maximum temperature. Instead, rainfall was shown to be the key factor correlating with fungal assemblages across the 12 sites. In forest health surveys that were conducted subsequent to this study (Wardlaw, unpublished data), serious levels of SNC were reported for two of the wettest sites only i.e. Inglis River and Oonah. These two sites in northwest Tasmania are in the same general area where a previous study found other plantations that had SNC (Podger & Wardlaw 1990. Podger and Wardlaw 1990 described SNC as being typical of a disease caused by a foliar pathogen that needs leaf wetness. However, they could not explain for inconsistencies to this theory such as low sensitivity to local topographic variation and year-to-year climatic fluctuation. Although SNC is not exclusive to high altitude sites, it is often seen at such sites (Wardlaw pers. comm.). *Pinus radiata* trees on high altitude sites with a marked difference between soil and air temperature in spring suffer physiological stress (Mohammed, unpublished data). Such a stress may potentially trigger *L. pinastri* into pathogenic activity. This study, conducted in plantations of an age that pre-dates the expression of SNC, showed that *L. pinastri* was ubiquitous in needles across all sites at which the climate is appropriate for stressful conditions to develop. One species, labelled Teratosphaeriaceae sp. 03, was present at only three of the 12 sites, but serious SNC subsequently developed at two of these sites. Another plausible scenario is that the causal agent of SNC is *L. pinastri* acting in conjunction with Teratosphaeriaceae sp. 03 when stressful conditions are present.

Podger and Wardlaw 1990 observed severe defoliation in the uppermost and well aerated parts of severely affected co-dominant trees. It could be that *L. pinastri* can act more as a primary pathogen than as an opportunistic. No detrimental effects of *L. pinastri* have been reported in New Zealand, but it may be that these isolates represent a different species to that found in Europe and Tasmania (Johnston et al 2003; Prihatini et al. 2014a). Tasmanian isolates had over 99% ITS sequence similarity to ATCC28347, designated by Minter et al (1978) as *L. pinastri*, whereas the New Zealand isolates had less than 95% similarity (Prihatini et al. 2014a). A taxonomic study including multigene phylogenetic analyses would assist in determining whether or not *L. pinastri* comprises of more than one species and may assist in clarifying the role of *L. pinastri* in needle cast diseases in *P. radiata*.

Outcomes of the thesis are as follows:

- **A reference collection and DNA database of *P. radiata* needle fungi has been established comprising of both endophytes and pathogens.** Isolates and the DNA database can be further mined to investigate the potential role of endophytes in needle cast diseases in Tasmania and elsewhere in the world, especially those that might be manipulated for biocontrol.
- **The taxonomy of the *Cyclaneusma* isolates present in Tasmania and New Zealand has been elucidated.** The differentiation of isolates into two species will support for the separate investigations of the pathogenic behaviour of each *Cyclaneusma* species and their interactions with endophytes. As the climate changes, the role or predominance of each species in causing disease may also change.

- **The causal agent of SNC in Tasmania is a ubiquitous needle fungus *L. pinastri,* which may act in conjunction with Teratosphaeriaceae sp. 03 when stressful conditions are present.** This knowledge establishes a causal agent for a disease (SNC) of previously unknown etiology and will support further investigations into this disease and its management.

Recommendations for future research are:

- Formal description of the new species of *Cyclaneusma.*
- The screening and testing of some of the endophytes detected e.g. either as candidate biocontrol agents for needle cast diseases or to understand the role of endophytes associated with diseased/declining trees.

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